

**Mechanisms involved in
retrotranslocation of ricin
from the endoplasmic reticulum
to the cytosol**

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Abbreviations

ADP	adenosine diphosphate
ARF	ADP-ribosylation factor
ATF6	activating transcription factor 6
ATP	adenosine triphosphate
BiP	binding protein
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CFTR	cystic fibrosis transmembrane conductance regulator
CIE	clathrin-independent endocytosis
CME	clathrin-mediated endocytosis
CNX	calnexin
COP	coatamer protein
CRT	calreticulin
dsRNA	double-stranded RNA
<i>E. coli</i>	<i>Escherichia coli</i>
EDEM	ER degradation enhancing α -mannosidase I-like protein
EE	early endosomes
eIF2	eukaryotic initiation factor 2
EM	electron microscopy
ER	endoplasmic reticulum

ERAD	ER-associated protein degradation
ERSE	ER stress response element
GI/GII	α -glucosidase I/II
HC	heavy chain
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
IRE	inositol requiring kinase
M6PR	mannose-6-phosphate receptor
MHC	major histocompatibility complex
mRNA	messenger RNA
NHK	null Hong Kong
PAGE	polyacrylamide gel-electrophoresis
PDI	protein disulfide isomerase
PERK	(PKR)-like ER kinase
PI	phosphatidylinositol
PKR	protein kinase R
PtdIns	phosphatidylinositol
PVDF	polyvinylidene fluoride
RIP	ribosome inactivating protein
RISC	RNA-induced silencing complex
RNA	ribonucleic acid

RNAi	RNA interference
rRNA	ribosomal RNA
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	sodium dodecyl sulfate
shRNA	small hairpin RNA
siRNA	short interfering RNA
SNX	sorting nexin
STEC	Shiga toxin-producing <i>E. coli</i>
sXBP1	spliced XBP1
TAP	transporter associated with antigen processing
TGN	<i>trans</i> -Golgi network
TPST	tyrosyl protein sulfotransferase
UDP	uridine diphosphate
UGGT	UDP-glucose:glycoprotein glucosyltransferase
UPR	unfolded protein response
XBP1	xbox-binding protein 1
Å	ångström

1. Introduction

An estimated 30% of all eukaryotic proteins misfold during their biogenesis in the endoplasmic reticulum (ER) [1]. Newly synthesised proteins are therefore subjected to a strict control machinery in the ER lumen where different chaperones facilitate folding during and after translocation [2]. If the folding process is incomplete, misfolded proteins are retrotranslocated (or dislocated) from the ER to the cytosol where they are finally degraded by the proteasomes. The process of selective protein export from the ER to the cytosol for proteasomal degradation is known as ER-associated degradation (ERAD) [3].

The Der1-protein of the budding yeast, *Saccharomyces cerevisiae* (*S. Cerevisiae*) has been shown to be required for retrotranslocation and degradation of a subset of ER substrates [4]. The mammalian genome contains three Der1-like proteins, named Derlin-1, -2 and -3. Derlin-1 was shown to be involved in degradation of class I major histocompatibility (MHC I) heavy chains (HCs) from the ER, catalysed by the human cytomegalovirus (HCMV)-encoded glycoprotein, US11 [5]. Furthermore, Derlin-1 promotes degradation of wild type and two common folding mutants of the cystic fibrosis transmembrane conductance regulator (CFTR) [6], and it was recently discovered that murine polyomavirus exits the ER membrane in a Derlin-2-dependent manner [7]. Overexpression of Derlin-2 and -3 was able to increase the degradation of the Null Hong Kong (NHK) mutant of the secretory glycoprotein α 1-antitrypsin [8]. Similarly, the inhibition of both proteins by gene-silencing blocked the degradation of this misfolded mutant [8]. Thus, the amount of substrates dependent on Derlin proteins for retrotranslocation to the cytosol is emerging.

The Derlin proteins may require additional factors to transport substrates across the ER-membrane. Experiments have shown that Derlin-1 and -2 forms a multiprotein complex with other components of the ERAD pathway [9]. It is not known whether the Derlin proteins themselves form a translocation channel or if they act as accessory proteins for another channel, such as Sec61.

The protein toxin ricin is a useful tool to study intracellular transport mechanisms. It consists of an A- and a B-chain that are linked together by a disulfide-bond. The B-chain is a lectin that binds to galactose-containing surface-receptors and the A-chain is able to bind to and inactivate ribosomes. The whole toxin is endocytosed and is transported in a retrograde manner via the *trans*-Golgi network (TGN) to the ER before it is retrotranslocated to the cytosol [10]. The disulfide-bond can be reduced by protein disulfide isomerase (PDI) and thioredoxin reductase in the ER [11,12]. It was proposed that this event partially unfolds the A-chain, making it able to cross the ER-membrane, presumably through the Sec61 translocon [13]. However, it has previously been shown that a ricin molecule with a non-reducible bond is equally cytotoxic as native ricin [14] indicating that intact ricin (holotoxin) might somehow reach the cytosol. Ricin holotoxin binds to both ER degradation enhancing α -mannosidase I-like protein (EDEM) and Sec61 [15] and preliminary data suggests that holotoxin is also able to reach the cytosol (Gregers et. al. unpublished data).

In the present study, we aimed to obtain new insight into the mechanisms involved in targeting of ricin to the cytosol. We have focused on the role of Derlin-3 in retrotranslocation of ricin, but have also addressed some questions regarding transport of holotoxin to the cytosol.

1.1 Entry of ricin into cells

1.1.1 Protein toxins

Many plants contain ribosome inactivating proteins (RIPs). Type I RIPs consist of a single ribosome-inactivating moiety. Absence of a binding moiety makes RIP I proteins relatively non-toxic. Type I RIPs include pokeweed antiviral protein, gelonin, trichosanthin and momordin [16]. Trichosanthin is one of the type I RIPs that

is toxic. It is isolated from the roots of *Trichosanthes kirilowii* and is in fact used to kill cells infected with human immunodeficiency virus [17].

Ricin is a type II RIP, meaning that it consists of an additional moiety that is able to bind to the cell surface. The combination of the two subunits makes RIP II proteins much more potent than type I RIP proteins [18,19]. Plants that produce type II RIPs include *Viscum album* (mistletoe), *Adenia Volkensii*, *Abrus precatorius*, and *Ricinus communis*.

Bacteria also produce RIP II proteins. Such bacterial toxins include Shiga toxin, secreted by *Shigella dysenteriae*, and Shiga-like toxins that are produced by *Escherichia coli* (*E. coli*) or other types of bacteria. Together with some bacterial toxins such as diphtheria toxin, cholera toxin and anthrax toxin, the RIP II proteins constitute the family of AB-toxins, which means that they have a binding moiety (B) that binds to the cell surface, and an enzymatically active moiety (A).

1.1.2 Why study protein toxins?

Protein toxins have provided knowledge in the field of endocytosis, intracellular transport routes and sorting mechanisms. For instance, the study of ricin gave some of the first evidence that there is more than one endocytic mechanism [20]. In addition, some of the first studies demonstrating recycling of endocytosed material was made by using ricin [21]. Furthermore, retrograde transport of cargo from the cell surface to the Golgi and the ER was first shown using Shiga toxin [22].

Several toxins are still a problem in connection with infectious diseases. For instance, *E. coli* producing Shiga-like toxins (STECs) is a problem even in developed countries. STEC disease is an increasing problem and when food borne outbreaks involve mass-distributed products, a lot of people might be affected. Once the toxin-producing bacteria have entered the body, they can cause severe diarrhea and renal

failure. Infants are especially susceptible to the toxin because they have more receptors on their kidney epithelial tissue than adults [23,24].

Protein toxins may be used as vectors to deliver peptides or epitopes into cells, that are to be presented by MHC I molecules on the cell surface [25-29]. The toxins are able to target specific cells and induce immunization without the need of an adjuvant. This can make them useful for vaccination purposes.

Toxins may also be used in targeted drug delivery, for instance by selectively killing cancer-cells. A toxin can be coupled to a targeting polypeptide, which may be an antibody or antibody fragment. Such a targeted toxin is called an immunotoxin and clinical trials are being performed [30,31].

Toxins are sometimes utilised in acts of bioterrorism, and knowledge about the effects on an organism can be vital.

1.1.3 Ricin

The plant toxin ricin is produced in the seeds of the castor-oil plant *Ricinus communis* (**figure 1**). Ingestion of the seeds may lead to vomiting and diarrhea, and in extreme cases death. The easy availability of ricin has made it a candidate for use as a homicidal weapon. The most famous incident includes the death of Georgi Markov, an exiled journalist who reported compromising information about the Bulgarian communist leadership. He was stabbed with an umbrella on September 7, 1978, and died a few days later. It was later revealed that the tip of the umbrella had been used to deliver a 1.52 mm diameter capsule that contained 0.2 mg of ricin to the subcutaneous tissue [32]. It is believed that the homicide was performed by the Bulgarian secret police. The same method was also used on Vladimir Kostov, another Bulgarian a month earlier. He luckily survived because the capsule had not yet dissolved in the tissue.

Ricin has also been used in several acts of terrorism. A recent episode involved a letter sent to the White House, which contained a powdery substance later identified as ricin. A person caught manufacturing or possessing ricin in the United States may be sentenced to up to 30 years in prison.



Figure 1: The castor-oil plant *Ricinus communis*. The picture is by courtesy of Professor Bo van Deurs.

Historical aspects

Ricinus communis is native to Asia and Africa, but now grows in all temperate and subtropical regions of the world. In some places the plant grows wild as a weed and it is even used as an ornamental plant because of its bright colours. The use of castor seeds in Egyptian and Greek folk medicine is described in the Sanskrit text *Susruta Ayurveda*. The castor-oil from the castor seeds can be extracted by pressurised heating and in this process, the toxin is destroyed. The oil is used in a modest amount in medicine as a laxative, but for the most part in industry as a lubricant [33].

As early as in 1888, Herman Stillmark discovered that the castor seed toxin was a protein, and he named it ricin [34,35]. He was able to purify the toxin and discovered that it induced blood-clotting. At first, it was believed that this was the property that caused the toxicity. Later, one discovered that the seeds contain strong agglutinins (*Ricinus* agglutinin) which are related to the toxin in structure, but are significantly less toxic.

Ricin remained largely forgotten by the scientific community until it was reported that the toxin acts on the protein synthesis machinery of the cell and that it could be useful in the treatment of cancer [36].

Many experiments were performed during the seventies, revealing among other things that the toxin is an enzyme [37,38] and that it consists of two polypeptide-chains linked together by a disulfide-bond [39]. The amino-acid sequence of ricin was determined by Funatsu et al. in 1979 [40] and was cloned by Butterworth and Lord in 1983 [41].

The crystal structure of ricin was first solved in the laboratory of Robertus in 1991 [42]. The smallest chain (32 kDa) was called A, and the largest (34 kDa) B. The B-chain is the binding moiety, whereas the A-chain is the enzymatically active part. **Figure 2** shows the crystallographic, together with a schematic, structure of ricin.



Figure 2: Crystallographic and schematic structure of ricin. Ricin consists of a 32 kDa A-chain (red) and a 34 kDa B-chain (green). The picture is obtained from [43].

Intracellular transport of ricin

Endocytosis

The B-chain of ricin is a lectin and is able to bind all over the cell surface because it interacts with glycolipids or glycoproteins with β -(1,4)-linked galactose residues [19]. After binding to the cell surface, ricin is endocytosed in both a clathrin-dependent and a clathrin-independent manner [44]. A significant amount of ricin is recycled from the endosomes back to the plasma-membrane [21]. The toxin is very resistant to proteolytic degradation, and only a small amount is degraded in the lysosomes.

Transport to the Golgi apparatus

About 5-10% of endocytosed ricin is transported to the TGN. The valency of the ligand seems to be important for sorting. Monovalent ricin-HRP reaches the Golgi apparatus, whereas multivalent ricin-HRP ends up in lysosomes [45].

Different transport machineries seem to be involved in transport of ricin to the Golgi apparatus. Ricin appears to be quite promiscuous, since several factors known to regulate retrograde transport of certain ligands, do not affect ricin trafficking. This is for instance the case for some of the members of the Rab-family of monomeric GTPases. Newly synthesised lysosomal enzymes bind to mannose-6-phosphate receptors (M6PRs) in the TGN, and are transported to the late endosomes where they are released. After this delivery, the M6PRs return to the TGN for another round of transport, and this return step is regulated by Rab9 [46,47]. In contrast to the M6PR, ricin is transported to the Golgi apparatus independently of Rab9 [48]. This Rab9-independency indicates that ricin utilises a direct route from early endosomes, circumventing late endosomes.

Phosphatidylinositols (PtdIns) are membrane phospholipids that can be phosphorylated by different kinases to form phosphoinositides (PIs). The phosphatidylinositol (PI) 3-kinase hVps34 phosphorylates PIs in the 3-position to form PI(3)P. hVps34 and its product PI(3)P have recently been shown to be involved in transport of ricin from the endosomes to the TGN [49]. The sorting nexin (SNX) family of proteins have PX domains that are able to bind to PI(3)P. SNX2 and SNX4

are effectors of the hVps34-dependent pathway and have been shown to be involved in endosome-to-Golgi transport of ricin [49].

Transport through the Golgi apparatus to the ER

After arrival at the Golgi apparatus, the toxin is transported in a retrograde manner to the ER. Retrograde transport of cargo through the Golgi apparatus can occur via coat-protein I (COPI) coated vesicles. The recruitment of a COPI-coat to a membrane is dependent on the GTPase ADP-ribosylation factor 1 (Arf-1). Retrograde cargo includes ER-resident proteins that contain the KDEL retrieval signal at their C-terminus. By interacting with the KDEL-receptor with different affinities in the Golgi apparatus and the ER, the proteins are released in the ER lumen. Cholera toxin has a KDEL-sequence and it was therefore thought that this would facilitate its COPI-coated vesicle transport to the ER [50,51]. However, cholera toxin is still able to reach the ER in the absence of the KDEL-sequence [52]. It was therefore suggested that the KDEL-sequence functions mainly to keep the toxin in the ER, and that the transport to the ER itself is not dependent on binding to the KDEL-receptor. Ricin does not have a KDEL-sequence, but it was hypothesised that it could hitch-hike with other molecules to gain access to the ER. A likely candidate was the ER-resident chaperone calreticulin which has galactose-residues to which ricin can bind, however calreticulin-deficient cell lines remained sensitive to ricin [53]. Inhibition of COPI or Arf-1 does not interfere with Golgi-to-ER transport of Shiga toxin or Shiga-like toxins [54] suggesting that there is another, COPI-independent transport pathway from the Golgi apparatus to the ER. Studies on Shiga-toxin suggests that this COPI-independent pathway is regulated by Rab6A [54,55]. However, ricin was still able to inhibit protein synthesis in cells where Rab6A and COPI were simultaneously inhibited [56], indicating that ricin utilises transport routes yet uncharacterised.

Translocation to the cytosol

Ricin exerts its toxic effect in the cytosol of cells, but how precisely the toxin exits the ER and translocate to the cytosol, remains unclear. However, the idea that protein toxins disguise themselves as misfolded proteins that are recognised as substrates for

ERAD is now widely accepted [57,58]. For ricin to exert its toxic effect, its A-chain must be reductively cleaved from its B-chain to release a steric block of the active site [59,60]. There is an abundance of redox-driven chaperones in the ER lumen and it has been reported that PDI [11] and thioredoxin reductase [12] is involved in ricin reduction. The reduction is thought to partially unfold the A-chain, rendering it competent to cross the ER-membrane [58]. Whether this reducing event is absolutely necessary for translocation to the cytosol remains unclear. Previously, a ricin molecule with a non-reducible bond was shown to be equally toxic to cells as native ricin [14], indicating that intact toxin might somehow be able to reach the cytosol.

EDEM, a component of the ERAD-machinery, was recently discovered to promote retrotranslocation of ricin from the ER to the cytosol [15]. Binding protein (BiP), an ER-luminal ATPase, is known to be involved in protein translocation from the ER [61]. BiP is required for transport of Cholera toxin [62] and binds Shiga toxin B-subunit [63], but its role in ricin transport is yet to be investigated. Ricin has been shown to interact with Sec61 [13] suggesting that the retrograde transport involves translocation through this pore. Recently, the Derlin family of proteins was suggested as another translocator. However, it remains unclear whether these proteins by themselves are able to form a pore, or if they simply work to facilitate transport through Sec61 [5]. The transporter associated with antigen processing (TAP-transporter) is another ER-membrane channel used by peptides. It transports peptides that are to be presented by MHC I molecules, from the cytosol into the ER [64]. Cells with a defective TAP-transporter are not resistant to ricin, indicating that ricin does not use this transporter to gain access to the cytosol [65]. Toxins that are transported from the ER to the cytosol have very low lysine content. Since lysine residues are sites for ubiquitination it is thought that these toxins have evolved this property to avoid degradation by the proteasomes [66].

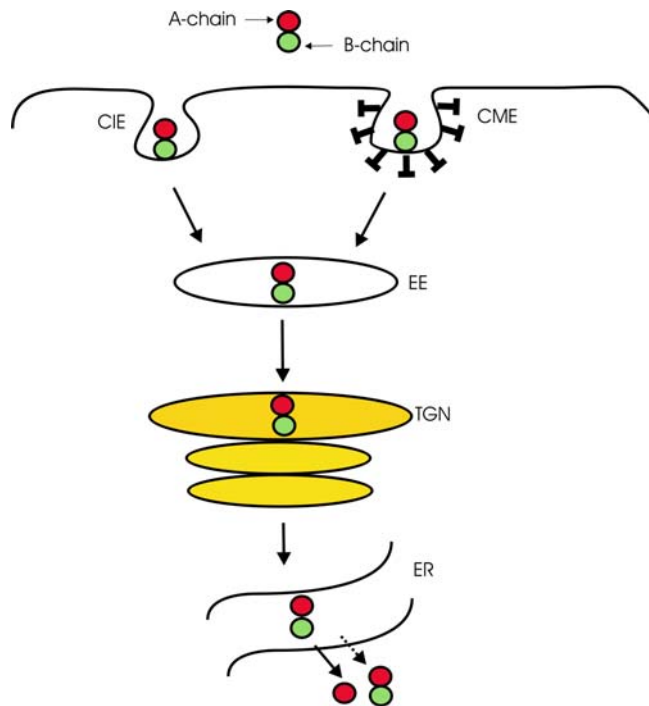


Figure 3: Intracellular transport of ricin. Ricin is endocytosed by clathrin-mediated endocytosis (CME) or clathrin-independent endocytosis (CIE), and is subsequently transported in a direct fashion from the early endosomes (EE) to the TGN and further to the ER. From here, the A-chain and perhaps also holotoxin is retrotranslocated to the cytosol.

Ricin toxicity

The ribosome-inactivating moiety of RIPs is a glycosidase that cleaves the *N*-glycosidic bond of an adenosine located in an exposed loop in the 28S ribosomal RNA (rRNA) fragment of the large ribosomal subunit [67]. This loop is critical for binding of elongation factors, and the modified ribosomes are unable to perform protein synthesis [68]. The mechanism of action is depicted in **figure 4**. Ribosome-inactivating toxins are very potent and can inactivate more than thousand ribosomes per minute [69]. They are able to inactivate ribosomes faster than an animal cell can make new ones and this eventually causes cell-death.

The toxin is then retrogradely transported to the ER before it is retrotranslocated to the cytosol. To measure toxin transport to the cytosol, cells are permeabilised using digitonin, a mild detergent. Digitonin binds specifically to cholesterol, forming an insoluble complex that makes permanent holes in the membrane [72]. These holes are about 8 nm in size and allow cytosolic proteins up to 285 kDa to leave the cell [73]. The large difference in cholesterol content of the plasma membrane and intracellular membranes allows selective lysis of the cholesterol-rich plasma membrane. If an appropriate concentration of digitonin is used, cholesterol-poor membranes will stay more or less intact. The fraction that leaks out upon digitonin-treatment is collected and referred to as the cytosolic fraction. The remaining fraction after lysis contains the membrane fraction.

Sulfated ricin sulf-1 is immunoprecipitated and separated using SDS polyacrylamide gel-electrophoresis (SDS PAGE). The gel is electroblotted onto an Immobilon polyvinylidene difluoride (PVDF) membrane. A radioactive-sensitive film is then placed onto the membrane to visualise radioactive ricin.

Transport to the ER

Translocation of ricin A-chain is proposed to occur from the ER, although attempts to visualise ricin in the ER have failed. Core glycosylation of proteins occurs in the ER lumen, and to study transport to this organelle, a modified ricin molecule, ricin sulf-2, was made [71]. This modified ricin molecule contains three partly overlapping *N*-glycosylation sites in the A-chain. Glycosylation of ricin increases its molecular weight so that it migrates slower in an SDS-polyacrylamide gel (**figure 5**). This makes it possible to separate ricin molecules that have reached the ER from those that have not.

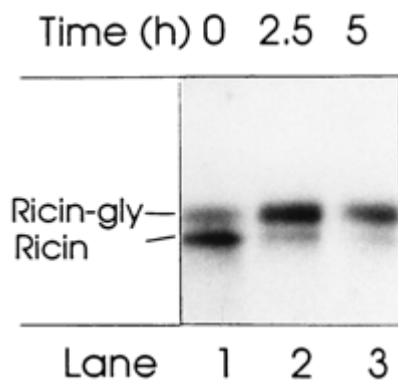


Figure 5: Time-course of glycosylation of sulfate-labelled ricin. Ricin sulf-2 contains *N*-glycosylation-sites in the A-chain. When the ricin sulf-2 molecule reaches the ER it becomes glycosylated, leading to an increase in molecular weight. Glycosylated ricin sulf-2 (Ricin-gly) migrates slower in an SDS-polyacrylamide gel than unmodified ricin (Ricin). The figure is obtained from [71].

Toxicity experiments

A toxicity assay can be used to study the transport of toxin to the cytosol. Cells are incubated in a leucine-free medium together with different dilutions of toxin ranging from 0.1-1000 ng/mL. After about 3 hours of incubation, the medium is replaced with [^3H]leucine-containing medium. Proteins that are synthesised after removal of toxin, will contain incorporated radioactive leucine. The amount of radioactively labelled proteins can then be measured using a β -counter, and a curve is plotted with protein synthesis in response to the amount of toxin added.

1.2 Quality control in the ER

Mammalian proteins are imported into the ER in a co-translational manner via the Sec61/ribosome complex. Sec61 is a multimeric complex consisting of an α -subunit spanning the membrane ten times and single-spanning β - and γ - subunits. The protein conducting channel is highly flexible, but still provides a barrier for ions and other small molecules. The permeability barrier is maintained by the tight binding of the ribosome to the ER membrane, which prevents small molecules from leaking out of the ER [74,75]. It is thought that BiP seals the pore on the luminal side of the ER

membrane by associating with ADP, and that the pore is re-opened when BiP substitutes ADP with ATP [76,77]. When visualised by electron microscopy (EM), Sec61 appears as a donut-like structure with stain-filled central cavities [78,79]. The actual pore-size has not yet been completely clarified, sizes from ~ 5 Ångström (or 0.5 nm) to as much as 60 Ångström have been suggested [80]. It is important to note that the channel was crystallised in its closed state. Therefore, the X-ray structure might not provide the complete truth about pore-size.

In the co-translational pathway, the channel binds to the translating ribosome. A multimeric enzyme called oligosaccharyltransferase (OST) transfers a core glycan composed of a glucose₃-mannose₉-N-acetylglucoseamine₂ structure (Glc₃Man₉GlcNac₂) to Asn-X-Ser/Thr motifs in the nascent chain [81]. The *N*-glycan property prevents aggregation of the peptide before it is correctly folded. The α -glucosidases I (GI) and II (GII) immediately remove two terminal glucoses from the polypeptide, so that it is able to interact with two other chaperones, calnexin (CNX) and calreticulin (CRT), each associated with ERp57. ERp57 catalyzes the formation of disulfide bonds between cysteine residues in the polypeptide chains. This is the rate-limiting step of polypeptide-folding in the ER. The folding polypeptide is eventually released, and the last remaining glucose is removed by GII. UDP-glucose:glycoprotein glucosyltransferase (UGGT) works as a folding sensor, scanning the polypeptide structure for non-native structures. If such structures are detected, UGGT adds a glucose-residue to the terminal mannose. This makes the polypeptide able to re-associate with CNX/CRT. Thus, the CNX/CRT chaperone system forms a cycle in which polypeptides can not escape until proper folding is achieved. When a protein is properly folded, it dissociates from CNX/CRT and is packed into secretory vesicles that exit the ER. However, not all proteins achieve the proper folding and these have to be disposed of by ERAD.

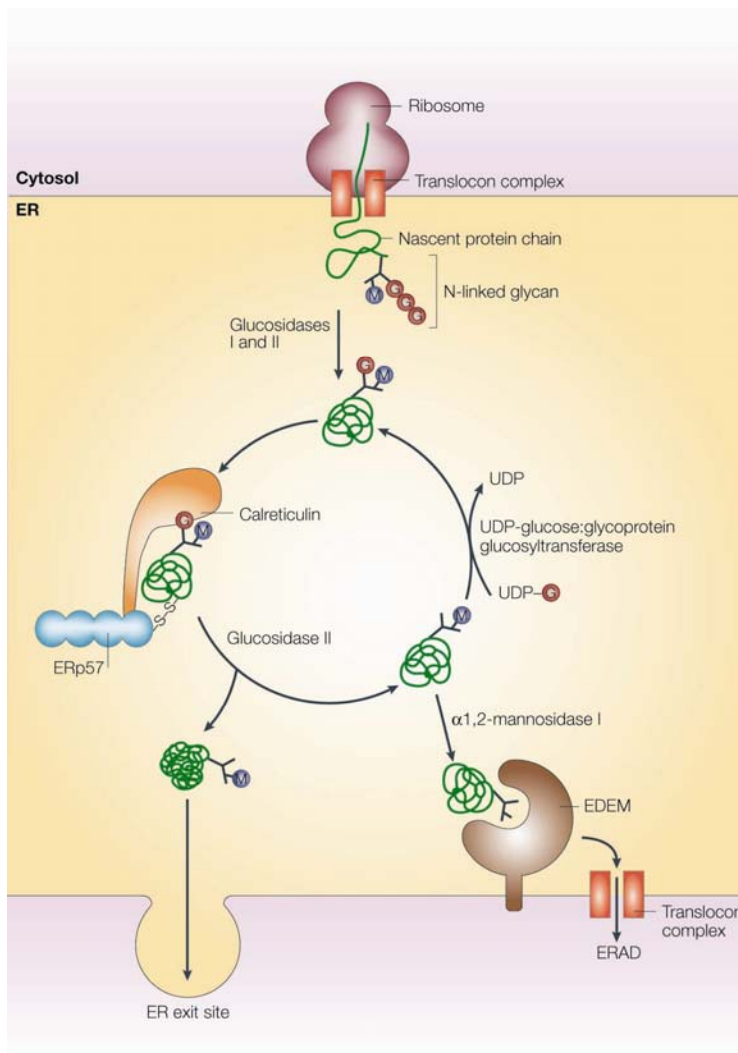


Figure 6: Quality control in the ER. Proteins are imported into the ER in a co-translational manner via the Sec61/ribosome complex. The CNX/CRT chaperone system forms a cycle in which polypeptides can not escape until proper folding is achieved. Properly folded proteins are transported from the ER through ER exit sites. Improperly folded proteins can interact with different chaperones that target them for degradation. The picture is obtained from [82].

1.2.1 ER-associated degradation (ERAD)

Degradation of proteins was previously thought to occur in the ER lumen [83]. Later came the discovery that misfolded secretory proteins are transported from the ER into the cytosol for degradation in the process now known as ERAD [3,84]. Proteins that are still not properly folded after participating in the CNX/CRT cycle, acts as targets for ER-mannosidase I, which removes a single mannosidase, creating a $\text{Man}_8\text{GlcNac}_2$

structure. It has been established that the formation of this structure acts as a signal for ERAD [85]. EDEM is thought to recognise this structure and target these proteins for degradation [86,87]. After a substrate has been targeted for degradation, it needs to be transported across the ER-membrane, presumably through the Sec61 translocon. Sec61 was first suggested to be involved in retrotranslocation, when it was observed that ERAD substrates were coimmunoprecipitated with this protein-conducting channel [88]. Genetic experiments also support a role of the Sec61 in retrotranslocation, in various yeast Sec61 mutants degradation of ERAD substrates is delayed or prevented [89-92].

Several ERAD substrates are polyubiquitylated at their cytosolic domain before complete export to the cytosol [93]. Attachment of the ubiquitin tag occurs in several steps. The first step includes the activating enzyme (E1), which attaches itself by a cysteine residue to the C-terminal carboxyl group of the small ubiquitin protein. The energy needed for this first reaction is provided by ATP hydrolysis. In the second step, ubiquitin is transferred to a cysteine residue in the conjugating enzyme (E2). The third step involves the transfer of ubiquitin to an amino-group of the substrate, often the ϵ -amino-group of a lysine, a process that requires ubiquitin-ligase (E3). Polyubiquitylation occurs by addition of several ubiquitin molecules to previously conjugated ubiquitin molecules [94]. Recent experiments indicate that this multiubiquitin chain is recognised by the cytosolic p97 AAA-ATPase, which pulls the substrate out of the ER-membrane [95-97]. The substrate is then bound to the proteasome, a large cage-like complex consisting of two subunits, a 20S proteolytic and a 19S regulatory subunit. The polyubiquitin chain is removed by isopeptidases before the substrate moves into the proteolytic chamber of the proteasome.

HCMV has been used to study ERAD in mammals, because it is able to take advantage of this pathway to avoid recognition by the immune system. It does so by destroying MHC I HCs. The HCMV genome encodes several proteins that are able to interfere with transport of MHC I products. Two of the immunoevasins encoded by the HCMV, US2 and US11, are able to catalyse dislocation of class I MHC HCs

[88,98]. Once the MHC HCs reach the cytosol, they are degraded by the proteasomes. This mechanism provides a way for the virus to avoid presentation of virus-encoded proteins on the cell surface and for the cell to be recognised by the cytotoxic T cells. The MKR protein of the murine γ -herpesvirus-68 works by a similar mechanism to destroy MHC I HCs [99], and the Vpu protein of the human immunodeficiency virus triggers ER-retention and degradation of the CD4 receptor [100].

The quality control mechanism of the ER and the degradation of improperly folded proteins are crucial for maintaining cellular homeostasis, but the stringent control mechanism can sometimes be detrimental. CFTR was the first integral mammalian protein to be implicated in ERAD [101,102]. CFTR has a very complex folding scheme. A substantial amount of the wild-type and nearly 100% of the most common mutant, the $\Delta F508$, is degraded by the proteasomes. The mutant protein might still be able to perform its function, but given the strict control machinery involved in folding of this important protein, it is not able to mature. Individuals that lack CFTR are unable to regulate Cl^- conductance in the apical membranes of airway epithelial cells [103,104]. Secondary events create inflammation and fibrosis of lung and pancreatic tissue.

However, the ER-quality control mechanism seems to be unable to deal with heavily aggregated proteins. More than 100 years ago, William Russell described intracellular structures now referred to as Russell bodies. They consist of dilated ER cisternae and contain aggregated immunoglobulins that have not been degraded. Misfolded influenza hemagglutinin can also form aggregates in the ER that are not degraded [105]. These aggregates do not cause cell-death, possibly because they do not expose hydrophobic patches that ER chaperones can bind to. They might be similar to inclusion bodies in the cytosol, which are also relatively benign.

1.2.2 The unfolded protein response

Accumulation of misfolded proteins in the ER triggers an unfolded protein response (UPR). The UPR has three major branches. The first involves increasing expression of house-keeping genes that are involved in proper protein folding. The second decreases the flow of secretory cargo into the ER and the third pathway increases the cells capacity for ERAD.

In mammals, there are three transmembrane proteins that acts as receptors for ER-stress, the inositol requiring kinase 1 (IRE1), double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) [106,107] (**figure 7**). These are kept in an inactive state through association with BiP. When misfolded proteins aggregate in the ER lumen, BiP dissociates from these receptors, and this triggers a signaling cascade that initiates the UPR. Once activated, PERK is able to phosphorylate eukaryotic initiation factor 2 (eIF2). This leads to inhibition of protein translation, which in turn leads to increased cell survival by decreasing the load of proteins to the ER. Activated ATF6 can be transported into the nucleus and induce genes that have an ER stress response element (ERSE) in their promoter. ATF6 also regulates the expression of Xbox-binding protein 1 (XBP1), another transcription factor. To achieve its active form, XBP1 undergoes mRNA splicing, which is carried out by IRE1. Spliced XBP1 protein (sXBP1) translocates into the nucleus and controls the expression of ER chaperones and genes involved in protein degradation (ERAD).

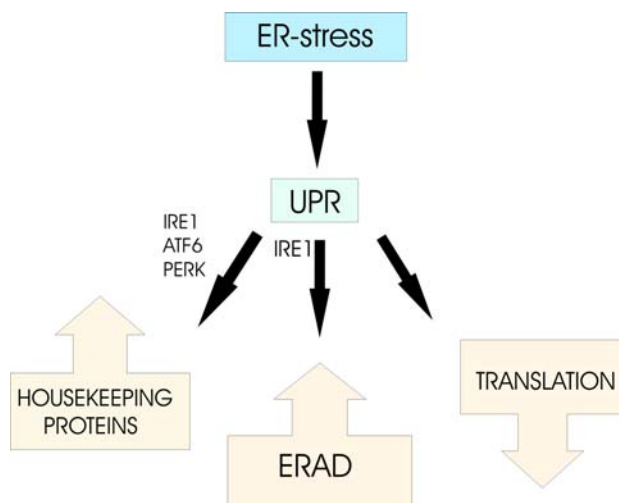


Figure 7: The unfolded protein response has three major pathways. First, the expression of housekeeping proteins is increased. Second, the flow of secretory cargo is decreased. Third, the capacity for ERAD is increased. There are three inducers of the UPR-signal, ATF6, IRE1 and PERK.

1.3 The Derlin-proteins

A protein of *S. cerevisiae* was shown to be required for degradation of a subset of ER substrates. It was named DER1, short for “degradation in the ER” [4]. A human homologue of this protein, Derlin-1, was demonstrated to cooperate with US11 in the dislocation of MHC I molecules. Derlin-1 recruits the p97 AAA-ATPase to the ER membrane, and is therefore thought to provide the missing link between events on the luminal side and on the cytosolic side of the ER membrane [5]. The US2 protein operates in a manner independent of Derlin-1, suggesting that several dislocation pathways exist.

Derlin-1 spans the ER membrane four times, with both the NH₂ and the COOH terminus facing the cytosol [5,108]. The mammalian genome encodes two more Derlin-proteins, Derlin-2 and 3, which both have the same topology as Derlin-1 [8]. Derlin-2 is a protein of 239 amino-acids that has 30% sequence similarity to Derlin-1. Derlin-3 has 70% sequence similarity to Derlin-2, and exists in two isoforms, denoted tv1 and tv2 (**figure 8**). These two isoforms are likely to be products of alternative

gene-splicing. Although mouse embryonic fibroblasts express only Derlin-2, all three are expressed in humans, but their expression varies between tissues.

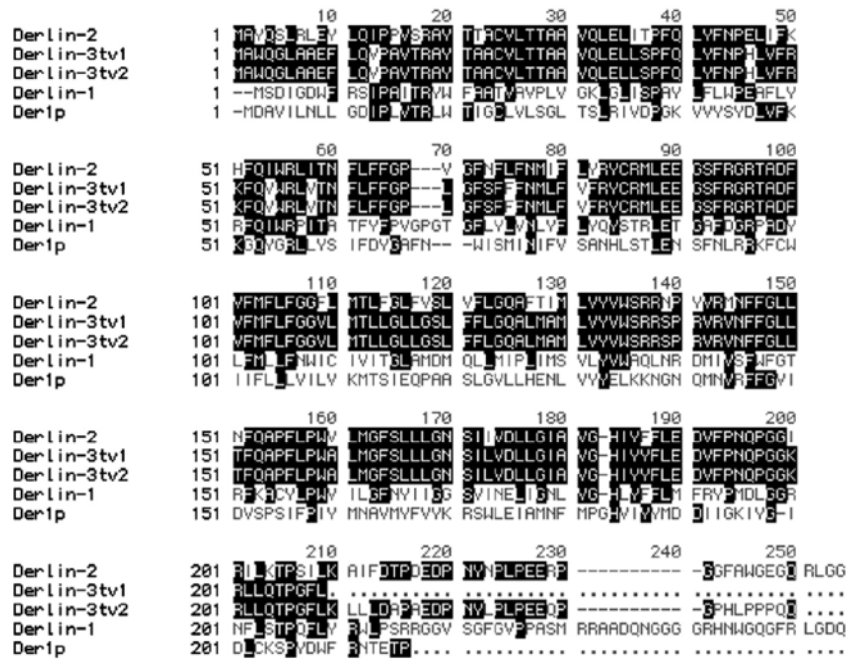


Figure 8: Amino acid sequence alignment of the Derlin proteins. The amino acid sequences of yeast Der1p and human Derlin-1, Derlin-2, Derlin-3 tv1 and Derlin-3 tv2 were compared. The alignment is obtained from [8].

The Derlin proteins may require additional factors in the retrotranslocation of misfolded proteins from the ER membrane. Experiments have shown that Derlin-1 and -2 form a multiprotein complex which includes the p97 AAA-ATPase and HRD1/SEL1 [9] (**figure 9**).

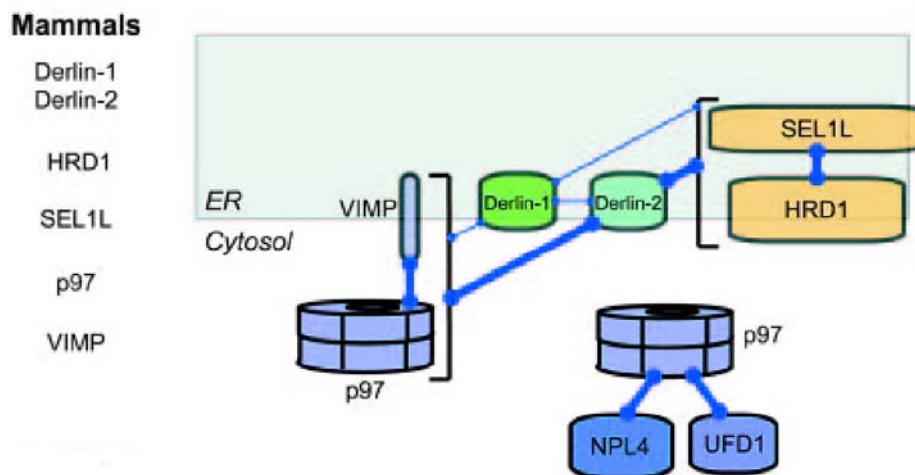


Figure 9: Derlin-1 and -2 form a multiprotein complex including the p97 AAA-ATPase and HRD1/SEL1. A cytosolic polyubiquitin is introduced onto emerging substrates. This polyubiquitin tag is recognised by the multiprotein complex which targets the substrate for degradation. The picture is obtained from [9].

The p97 AAA-ATPase is together with its cofactors Ufd1 and Npl4, involved in pulling out ubiquitylated proteins from the ER and transferring them to the cytosol for degradation by the proteasomes [109]. Hrd1 is an ER membrane protein that acts as an E3 ubiquitin ligase and is involved in degradation of ER proteins to protect the cell from ER stress-induced apoptosis [110,111]. VIMP (also known as selenoprotein S [112] or Tanis [113]) is a receptor in the ER membrane for the p97 AAA-ATPase complex, but its role in protein dislocation is unclear [108]. SEL1 is thought to be involved in substrate recognition of misfolded proteins in the ER and in directing them to the site of dislocation [114]. The complex might form a link between movement of substrate across the ER membrane, and ubiquitination and extraction. The mechanism of action is proposed in **figure 10**.

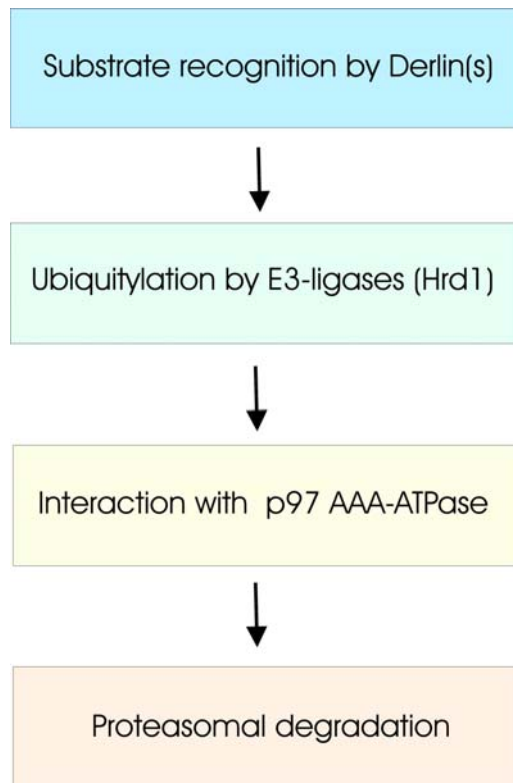


Figure 10: Mechanism of action of the Derlin-dependent ERAD pathway.

Misfolded substrates are recognised by the Derlin proteins before they are ubiquitylated and pulled out of the ER membrane by the p97 AAA-ATPase, to be degraded by the proteasomes.

When folding and ER export of CFTR is unsuccessful, ubiquitin ligases must be recruited to promote degradation of the substrate [115,116]. Derlin-1 was shown to promote degradation of two CFTR folding mutants, $\Delta F508$ and G85E. It is proposed that the initial interaction with Derlin-1 is followed by CFTR ubiquitylation and dislocation from the ER by the p97 AAA-ATPase [6].

Murine polyomavirus is transported to the ER and is altered before the virus particle exits the ER-membrane and is transported into the nucleus. Derlin-2 is involved in the escape of the virus from the ER. Thus, Derlin proteins are utilised by at least two families of viruses for different purposes: (i) immune evasion in one case (HCMV US11 and Derlin-1) (ii) and viral exit from the ER in the other (polyomavirus and Derlin-2) [7].

The protein levels of both EDEM and the Derlin proteins have been shown to increase upon ER stress. The transcriptional induction of EDEM and the Derlin

proteins in response to ER stress is dependent on the IRE/XBP1 pathway [8,117]. A commonly used substrate to study ERAD mechanisms is the NHK mutant of the secretory glycoprotein α 1-antitrypsin. NHK that is glycosylated and misfolded in the ER lumen is recognised by EDEM and targeted for destruction in the proteasomes [85]. Overexpression of Derlin-2 and -3 was able to increase NHK degradation. Similarly, decreased expression of Derlin-2 and -3 by RNAi blocked the degradation of this misfolded mutant. Experiments on overexpression of Derlin-2 and -3 were also performed on a modified NHK, designated NHK(QQQ), where all asparagines of three possible *N*-glycosylation were substituted with glutamine. Overexpression of Derlin-2 and -3 did not accelerate degradation of this mutant. Taken together, these results indicate that Derlin-2 and -3 assist in degradation of misfolded glycoproteins, but not of non-glycoproteins [8].

Derlin-2 and -3, but not Derlin-1, are associated with EDEM indicating a functional difference between Derlin-1 and a group consisting of Derlin-2 and -3. Furthermore, Derlin-3 was coimmunoprecipitated with Derlin-2, but was shown to be poorly associated with Derlin-1. Derlin-2 and -3 are associated with the degradation substrate, p97 AAA-ATPase and EDEM, and thereby provide a link between EDEM and p97 AAA-ATPase in the degradation of misfolded glycoproteins [8].

When expressed simultaneously, Derlin-2 and -3 are able to form heterooligomers. Similarly, they may form homooligomers when expressed singularly [8]. It is of great interest to know whether misfolded glycoproteins might be translocated through a channel formed by Derlin-2 and -3, as has been proposed for Derlin-1 [5,108].

Transport of cargo from the ER to the Golgi apparatus is mediated by COPII-coated vesicles [118]. Recently, a vesicular budding pathway out of the ER was discovered, that did not involve COPII-coated vesicles [119]. EDEM was found to be sequestered into these vesicles, and Derlin-2 and NHK was also found here [119]. These vesicles could provide an additional pathway for clearance of misfolded proteins from the ER.

It seems more than one pathway out of the ER exist, and it is possible that different ERAD substrates use different translocation-mechanisms. Thus, the events occurring in the ER-lumen and how they are connected to those in the cytosol need to be further investigated.

1.3.1 Methods to study the role of Derlin-3

Transfection

One can make a cell overexpress a certain protein by inserting the gene of interest into a plasmid vector which is then introduced into the host cell. The plasmid may typically contain a strong promoter sequence, such as the CMV-promoter. An efficient method to introduce the foreign DNA into the cell is transfection by help of liposomal agents which fuse with the membrane and release the vector into the cell. Expression of the transfected gene is usually transient, which means that the foreign DNA is not incorporated into the host genome and is therefore lost during cell-division.

RNA interference

RNAi is a method for knocking down target mRNA in an organism, leading to silencing of the gene product. The phenomenon was first discovered in *Caenorhabditis elegans*, where it was noted that introducing a double-stranded RNA (dsRNA) that was homologous to a specific gene, resulted in the post-transcriptional silencing of that gene [120]. The mechanism of action has now been elucidated and consists of two main steps: First, the dsRNA is recognised by Dicer, an enzyme of the RNaseIII family of endonucleases [121]. Dicer will cleave the dsRNA into smaller, double-stranded fragments, referred to as siRNAs. Second, the siRNAs are incorporated into the RNA-induced silencing complex (RISC) which unwinds the siRNA duplex in a process requiring ATP. Once unwound, the single-stranded antisense strand guides RISC to a complementary strand of mRNA which is then

cleaved into smaller pieces. The cleaved mRNA no longer gives any functional gene product. This eventually leads to a successful downregulation of the protein of interest, depending on the half-life of the protein. There are two main approaches for gene-silencing through RNA-interference, namely the use of synthetic short siRNAs or the vector-based approach where the sequence is cloned into a plasmid vector and expressed through a strong promoter.

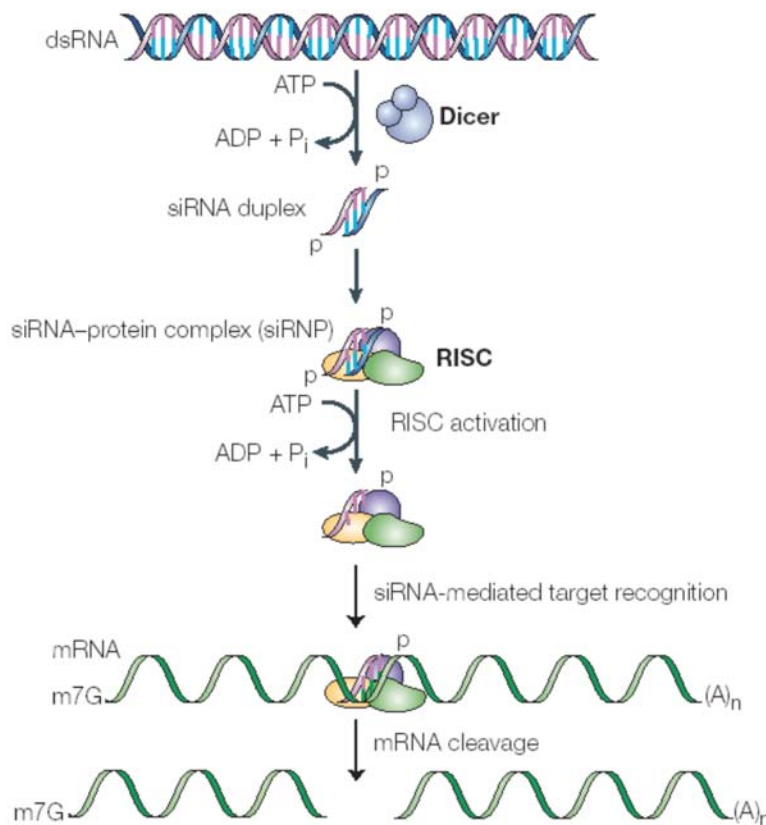


Figure 11: Gene silencing using RNAi. dsRNA is recognised by Dicer and cleaved into smaller pieces. The resulting siRNAs will then bind to RISC which is targeted to a complementary strand of mRNA. The target mRNA is then cleaved and this results in gene silencing. The picture is obtained from [122].

When dsRNA of more than 30 base pairs in length was used in mammalian cells, it was found that there was an inhibition of translation because of the interferon (IFN) system that is part of the body's defence against viral infection [123]. A method was therefore developed that included the use of synthetic 21-23 base pair siRNAs that bypass the initial Dicer step, but still maintain the ability to bind to RISC. The design of siRNAs involves choosing a target sequence of about 21 nucleotides long that

fulfils certain requirements [124]. During the last years, the use of synthetic siRNAs has been the most common method for gene silencing in mammalian cells. These synthetic siRNAs can be transfected directly using a liposomal reagent.

More recently, plasmid- and viral-based expression of small hairpin RNAs (shRNA) has been developed. The vector-based method involves expression of the siRNA from a strong promoter sequence in a plasmid vector. The use of vector-based siRNA may be more efficient than synthetic siRNA, since they are continuously expressed. In the U6-siRNA PCR amplification approach, a primer is designed that encodes the sense and antisense strands of the target sequence separated by a loop sequence and flanked by a restriction site. In addition, the primer also contains a short sequence complementary to the U6 promoter. A PCR is then performed with a second primer that has another restriction-site and with a vector containing the U6 promoter as a template. The resulting product is then cloned into the same expression vector (**figure 12**).

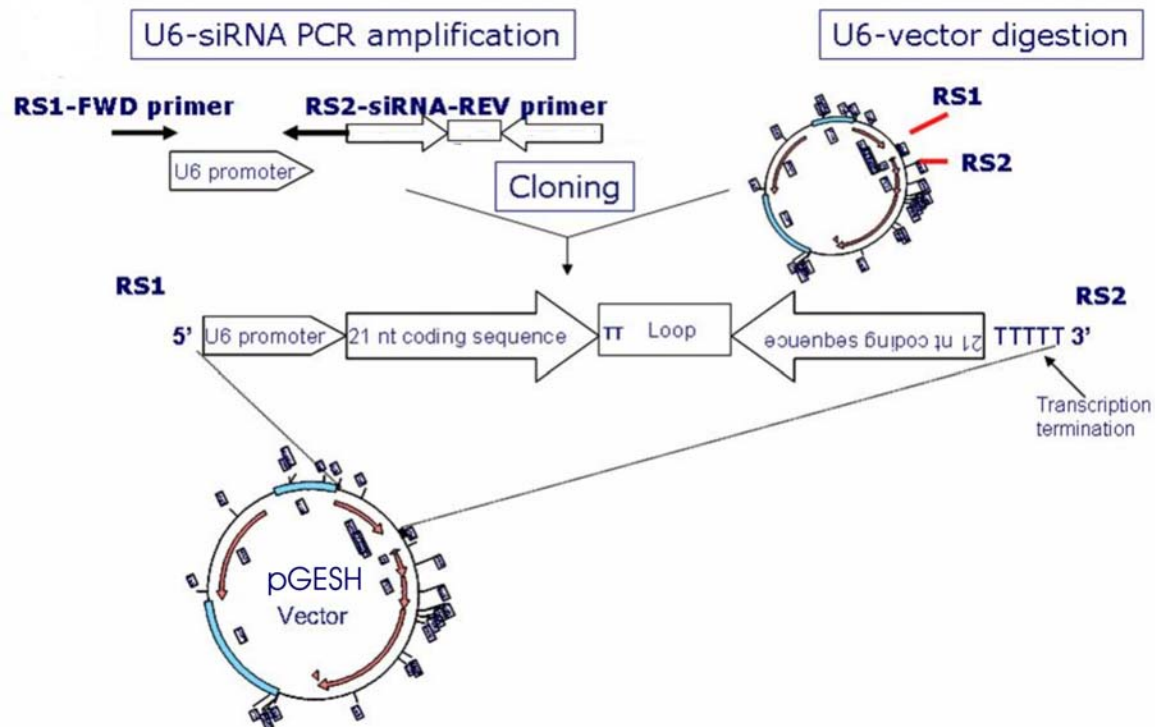


Figure 12: The vector-based approach to RNA-interference. The vector-based method involves expression of the siRNA from a strong promoter sequence in a plasmid vector. This allows for continuous expression of siRNA.

Aims of the present study

Several protein toxins have cytosolic targets, and study of these toxins has provided knowledge about mechanisms of endocytosis and intracellular transport. Ricin follows a retrograde pathway from the plasma membrane through *trans*-Golgi network and into the ER before retrotranslocation to the cytosol.

Proteins that are misfolded in the ER are retrotranslocated into the cytosol for degradation in a process now known as ERAD. Ricin is thought to disguise itself as a misfolded protein that is recognised as substrate for ERAD in order to gain access to the cytosol. The first manuscript addresses the question of whether Derlin-3, a component of the ERAD machinery, is involved in retrotranslocation of ricin from the ER to the cytosol.

Ricin consists of an A- and a B-chain linked together by a disulfide-bond. The B-chain is recognised by receptors on the surface of the target cell, while the A-chain exerts the toxic effect. Ricin can be reduced in the ER before the A-chain is retrotranslocated to the cytosol. Recently, also holotoxin has been visualised in the cytosol (Gregers et. al. unpublished results). Thus, we have also studied retrotranslocation of holotoxin from the ER to the cytosol and whether this requires prior reduction of the disulfide-bond. This study is part of a manuscript in preparation (Gregers et. al, “Retrotranslocation of ricin holotoxin from the endoplasmic reticulum the cytosol”) and is presented in Appendix I.

Perspectives

The ER is a fine-tuned machinery of chaperones and oxidoreductases that ensures proper folding and oligomerisation of proteins. Exactly how improperly folded proteins are recognised by the ERAD machinery and which by which mechanisms they translocate across the ER-membrane is a field of much interest.

Protein toxins have provided us with knowledge of endocytosis and mechanisms of intracellular transport. The protein toxin ricin is able to exploit the ERAD machinery to gain access to the cytosol. The use of ricin therefore provides a way to study these mechanisms further

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2. Manuscript

THE ROLE OF DERLIN-3 IN RETROTRANSLOCATION OF RICIN FROM THE ENDOPLASMIC RETICULUM TO THE CYTOSOL

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Abstract

Ricin is a plant toxin produced in the seeds of the castor oil plant *Ricinus communis*. After endocytosis, the toxin is transported in a retrograde manner to the endoplasmic reticulum (ER) via the *trans*-Golgi Network (TGN) before it is retrotranslocated to the cytosol where it exerts its cytotoxic effect by inhibiting protein synthesis. Proteins that are misfolded in the ER are transported to the cytosol for degradation in a process known as ER-associated degradation (ERAD). Ricin is thought to exploit this mechanism to gain access to the cytosol. The mammalian Derlin-proteins are components of the ERAD machinery, and Derlin-3 has been shown to promote the retrotranslocation of misfolded glycoproteins to the cytosol for degradation. In the present study, we have investigated the role of Derlin-3 in retrotranslocation of ricin from the ER to the cytosol by performing overexpression and RNA interference (RNAi) studies.

Introduction

The endoplasmic reticulum (ER) represents the origin of the secretory pathway. In eukaryotes, proteins are transported in a cotranslational manner into the ER through a narrow pore in the membrane known as the Sec61 translocon that is closely associated with the ribosomes. In the ER lumen, nascent polypeptides are subjected to chaperones that assist in folding and oligomerisation. In spite of the stringent control machinery, an estimated 30% of all eukaryotic proteins misfold during their biogenesis in the ER. These misfolded proteins can form harmful aggregates and need to be disposed of by the cell. It was previously thought that faulty proteins were degraded in the ER lumen [1], but it is now known that this occurs in the cytosol where they are degraded by the proteasomes. The transport of misfolded proteins from the ER into the cytosol for degradation is known as ER-associated degradation (ERAD) [2,3].

Studies on the budding yeast, *Saccharomyces cerevisiae* revealed a hydrophobic protein required for the degradation of a subset of ER substrates [4]. The protein was named DER1, an abbreviation for ‘degradation in the ER’. The mammalian genome contains three DER1-like proteins named Derlin-1, -2 and -3. The human cytomegalovirus (HCMV)-encoded glycoprotein, US11, cooperates with Derlin-1 to promote retrotranslocation of class I major histocompatibility (MHC I) heavy chains (HCs) from the ER to the cytosol [5]. Other viruses may also exploit the ERAD pathway. Murine polyomavirus is transported to the ER and is retrotranslocated in a Derlin-2 dependent manner [6]. Cystic fibrosis transmembrane conductance regulator (CFTR) was the first integral membrane mammalian protein found to be a substrate for ERAD [7,8]. Derlin-1 interacts with both wild type and two CFTR folding mutants and promotes their degradation [9].

The null Hong Kong (NHK) mutant of the secretory glycoprotein α 1-antitrypsin is a commonly used substrate to study ERAD function. The mutant protein that is terminally misfolded in the ER lumen, is recognised by ER degradation enhancing

α -mannosidase I-like protein (EDEM) and targeted to the proteasomes for degradation [10]. Overexpression of Derlin-2 and -3 was able to increase the degradation of NHK. Similarly, the inhibition of both proteins by RNAi blocked the degradation of this misfolded mutant [11]. Thus, the amount of substrates dependent on Derlin proteins for retrotranslocation to the cytosol is emerging.

Ricin is a ribosome inactivating protein (RIP) produced in the seeds of the castor oil plant *Ricinus communis*. It consists of two subunits, an A- (32 Kda) and a B-chain (34 kDa) that are held together by a disulfide-bond. The B-chain is a lectin and is able to bind to cell-surface receptors with galactose-residues [12]. After binding, the toxin is endocytosed in both a clathrin-dependent and a clathrin-independent manner [13]. A significant amount of the toxin is recycled back to the plasma membrane after endocytosis [14], and a large part ends up in the lysosomes for degradation. About 10-15% is transported retrogradely via the *trans*-Golgi network (TGN) to the ER. It has been reported that protein disulfide isomerase (PDI) [15] and thioredoxin reductase [16] is able to reduce ricin in the ER and it is thought that this reducing event partially unfolds the A-chain rendering it competent to cross the ER membrane to the cytosol. When the A-chain reaches the cytosol it is able to inactivate ribosomal protein synthesis by removing an adenine from an exposed loop in the 28S ribosomal RNA of the 60S ribosomal subunit [17].

Protein toxins have proven to be useful tools to study endocytosis and intracellular transport. The toxins are thought to exploit the ERAD machinery to gain access to the cytosol [18,19]. Thus, we have studied the involvement of Derlin-3 in retrotranslocation of ricin by using overexpression and RNA interference (RNAi) of this protein in HEK-cells.

Materials and methods

Materials

Reagents and inhibitors: Ricin, Hepes, lactose and digitonin were all obtained from Sigma Chemical Co. (St Louis, MO, USA). [³H]leucine was purchased from GE Healthcare (Princeton, NJ, USA). Na₂³⁵SO₄ is manufactured by Montebello diagnostics (Montebello diagnostics, 0379 Oslo, Norway).

Antibodies: Rabbit polyclonal anti-ricin antibodies were from Sigma Chemical Co (St Louis, MO, USA). Mouse anti-c-myc antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) were used for Western immunoblotting and confocal-microscopy studies. Mouse anti-γ-tubulin antibodies (Sigma Chemical Co., St Louis, MO, USA) were used for Western immunoblotting. Rabbit anti-PDI antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) were used in confocal microscopy-studies. Horseradish peroxidase-linked goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary antibody in Western immunoblotting.

Restriction enzymes: All restriction enzymes (*Bgl*III, *Kpn*I and *Xho*I), and the T4 DNA ligase were from New England Biolabs (Beverly, MA, USA).

Cell culture

Human Embryonic Kidney-cells (HEK 293) are an epithelial cell-line originally derived from embryonic kidney. These cells were used in all experiments. Cells were grown in Falcon cell culture flasks (BD Biosciences, Palo Alto, CA, USA) and maintained in Dulbecco's Modified Eagle Medium DMEM (BioWhittaker, MD, USA) supplemented with 10% fetal calf serum (FCS) (Integro b.v, Zaandam, Netherlands), 100 units/mL penicillin and 100μg/mL streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a 5% CO₂ atmosphere. Cells were seeded on Falcon cell culture plates coated with poly-L-lysine (Sigma Chemical, St Louis, MO).

Vector-based siRNAs

Vector-based siRNAs were made using a pGESH shuttle-vector containing a U6-promoter. Two sequences were selected for targeting knock-down of Derlin-3. Both sequences were checked for specificity using the Basic Logic Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database.

For each sequence, a primer was designed that contained the sequence in sense and antisense direction (bold).

Primer #1 had the sequence

5'- ACTAGTCTCGAGAAAAA**AGCGTCCTTATGACCCTGCTGGGGTACC**
GAACAGCAGGGTCATAACGCGGATCCTCGTCCTTTCCACAAGATAT-3'

and primer #2 had the sequence

5'- ACTAGTCTCGAGAAAAA**AGGCTCGTCACCAACTTCCTCGGGTACC**
GAAGAGGAAGTTGGTGACGAGCCTGGATCCTCGTCCTTTCCACAAGATAT-3'.

In addition, the primers had a short stretch complementary to the U6 promoter and a 5' end containing an *XhoI* restriction site (italic) and a *KpnI* restriction site (underlined). A PCR was performed using each of the siRNA primers separately together with universal primer containing a *BglIII* restriction site and an empty pGESH vector as template DNA. The resulting PCR-product forms a siRNA construct with a hairpin loop containing a *KpnI* restriction site. The vector and the siRNA constructs were then cut separately with the restriction enzymes *BglIII* and *XhoI*, before they were ligated together to form a plasmid expressing the siRNA via the U6 promoter. A test-cut to check for insert of the siRNA was performed with *KpnI*. The constructs were sequenced.

Oligo-based siRNAs

For the knock-down of Derlin-3 using the synthetic siRNA approach, two 21 nucleotide sections of the genome sequence was selected; siRNA 3,

5'- AGGCUCGUCACCAACUUCCUCTT – 3'

and siRNA 4,

5'- AACUCUACUUCAACCCGCACCTT -3'

Both sequences were checked for specificity using the Basic Logic Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database.

DNA constructs

Plasmids expressing both transcription variants of Derlin-3 were a generous gift from the laboratory of Prof. Kazutoshi Mori (Department of Biophysics, Kyoto University, Japan). The plasmids were pCDNA4 vectors that expressed Derlin-3 cDNA cloned as a C-terminal fusion myc from a CMV-promoter.

Transient transfection

Transient transfection of synthetic siRNA was done using OligofectamineTM reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. A concentration of 100nM oligo was used. Cells were incubated with the transfection-mix for two days before performing experiments.

Transient transfection of the vector-based siRNA and the Der-3-myc constructs was done using the FuGENE6® reagent (Roche diagnostics, Mannheim, Germany) according to the protocol. Experiments were performed two days post-transfection.

Western immunoblot analysis

Cells were washed twice with cold PBS before lysis in lysis-buffer (0.1M NaCl, 10mM Na₂HPO₄, 1mM EDTA and 1% Triton X-100, pH 7.4) supplemented with complete protease inhibitors (Roche Diagnostics, Basel, Switzerland). Lysate was harvested with a cell-scraper and transferred to eppendorf-tubes before centrifugation for 5 minutes at 4000 rpm at 4°C to remove cell debris. The supernatant was mixed with a reducing buffer and boiled for 5 minutes at 100°C. Proteins were then analyzed by 12% SDS-PAGE and subsequently electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked using PBS/0.05% Tween-20 with 5% of non-fat dried milk. All antibodies were diluted in 5% non-fat dried milk in PBS with 0.1% Tween-20. SuperSignal West Pico Chemiluminescent substrate and SuperSignal West Femto Chemiluminescent substrate (Pierce, Rockford, IL) was used for immunodetection.

Confocal microscopy studies

Cells were grown on coverslips and transfected with the Derlin-3-myc plasmids. The cells were fixed using a 10% formalin solution (Sigma-Aldrich, Stenheim Germany) 24 hours post-transfection. Lysis of the cells was done using 0.2% Triton X-100:PBS for 10 minutes. After lysis, a blocking solution was applied consisting of 5% FCS in PBS for 30 minutes. Transfected cells were identified using mouse anti-myc antibodies followed by rhodamine-labelled anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

The ER was labelled with a rabbit anti-PDI antibody (Santa Cruz Biotechnology, Heidelberg, Germany) and visualised with a CY2-labeled anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The coverslips were mounted on micro-slides with Mowiol (Calbiochem, San Diego, CA). The slides were dried for 15 minutes at 37°C and then analyzed with a Zeiss LSM510 META confocal microscope (Zeiss, Germany). Images were processed with Zeiss LSM Image Browser version 4.0.

Ricin toxicity and measurement of protein synthesis

Cells were seeded on 24-well plates with a density of 3×10^4 cells/well and transfected with an empty control vector and the appropriate construct. Two days post-transfection, cells were washed in a leucine-free medium and incubated with different concentrations of ricin toxin for 3 hours at 37°C. The medium was removed and cells were incubated for 20 minutes with a medium containing 1 μ Ci/mL [3 H]leucine at 37°C. The medium was then removed and the cells were washed with 5% trichloroacetic acid (TCA). Acid precipitable proteins were dissolved in 0.1M KOH before amount of incorporated [3 H]leucine in newly synthesised proteins was measured on a Packard Tri-Carb Liquid Scintillation Analyzer 2100TR β -counter (PerkinElmer).

Sulfation of ricin sulf-1 and permeabilisation of cells

Ricin sulf-1 contains a sulfation site that can be utilised to measure transport to the Golgi apparatus. Ricin A-chain sulf-1 was produced, purified and reconstituted with ricin B-chain to form ricin sulf-1 [20]. It is known that after approximately 3 hours, the toxin reaches the ER where it is retrotranslocated to the cytosol. To measure transport to the cytosol, cells are treated with low concentrations of digitonin to permeabilise the plasma membrane, allowing cytosolic proteins to leak out.

Cells were incubated for 3 hours in sulfate-free medium containing 1% non-essential amino-acids (Bio Whittaker) and 0.2mCi/mL $\text{Na}_2^{35}\text{SO}_4$ before $\sim 500\text{ng/mL}$ ricin-sulf-1 was added, and the incubation was continued for 3 hours at 37°C. The medium was removed, and cells were washed twice at 37°C with a solution of 0.1M lactose in HEPES-medium. Cells were then washed with room-tempered PBS and incubated with a KOAc-buffer (115 mM CH_3COOK , 25 mM HEPES, and 2.5mM MgCl_2 , pH 7.4) containing 3 $\mu\text{g/mL}$ digitonin followed by 30 a minute incubation on ice allowing cytosolic proteins to leak out of the cells. The cytosolic fraction was collected and lysis-buffer (0.1M NaCl, 10mM Na_2HPO_4 , 1mM EDTA and 1% Triton X-100, pH 7.4) in the presence of a protease inhibitor (Roche diagnostics, Mannheim, Germany)

was added to the remaining fraction. Both fractions were spun down for 5 minutes at 5000 rpm in an eppendorf centrifuge (Eppendorf, Hamburg, Germany) to remove debris and nuclei.

Immunoprecipitation of ricin sulf-1 was done using protein-A-sepharose CL-4B (GE Healthcare, Princeton, NJ, USA) coated with rabbit-anti-ricin antibodies overnight at 4°C. After binding of sulfated ricin, the beads were washed with a 0.35% Triton X-100: PBS solution. A non-reducing loading buffer was then added to the beads before boiling at 100 °C to release bound material. SDS-PAGE was performed using a 12% gel, and the proteins were electroblotted onto a PVDF membrane (Millipore, Billerica, MA, USA). Dried membranes were exposed to Kodak BioMax MR film (Rochester, NY, USA) at room temperature. The intensities of the bands were quantified using ImageQuant Tool version 5.0 (GE Healthcare, Princeton, NJ, USA). The slowest migrating band of 60 kDa represents intact toxin (holotoxin), and the fastest migrating band of 30 kDa is ricin A-chain.

Cell lysates were precipitated in 5% TCA and dissolved in 0.1M of KOH before the radioactivity was measured on a β -counter. The radioactivity measured in the precipitates represents total protein sulfation. This is done as a control to detect possible differences in the total amount of isotope incorporated under the different conditions.

Results

Visualization of Derlin-3 using confocal microscopy studies

To confirm the localization of Derlin-3 and to check the expression of the Derlin-3-myc cDNA plasmids (See Materials and Methods), we used confocal microscopy studies. Cells were transfected with plasmids expressing Derlin-3-myc transcription variant 1 and 2 (tv1 and tv2). Confocal microscopy was performed with anti-myc antibodies to visualise Derlin-3-myc and anti-PDI antibodies to label the ER.

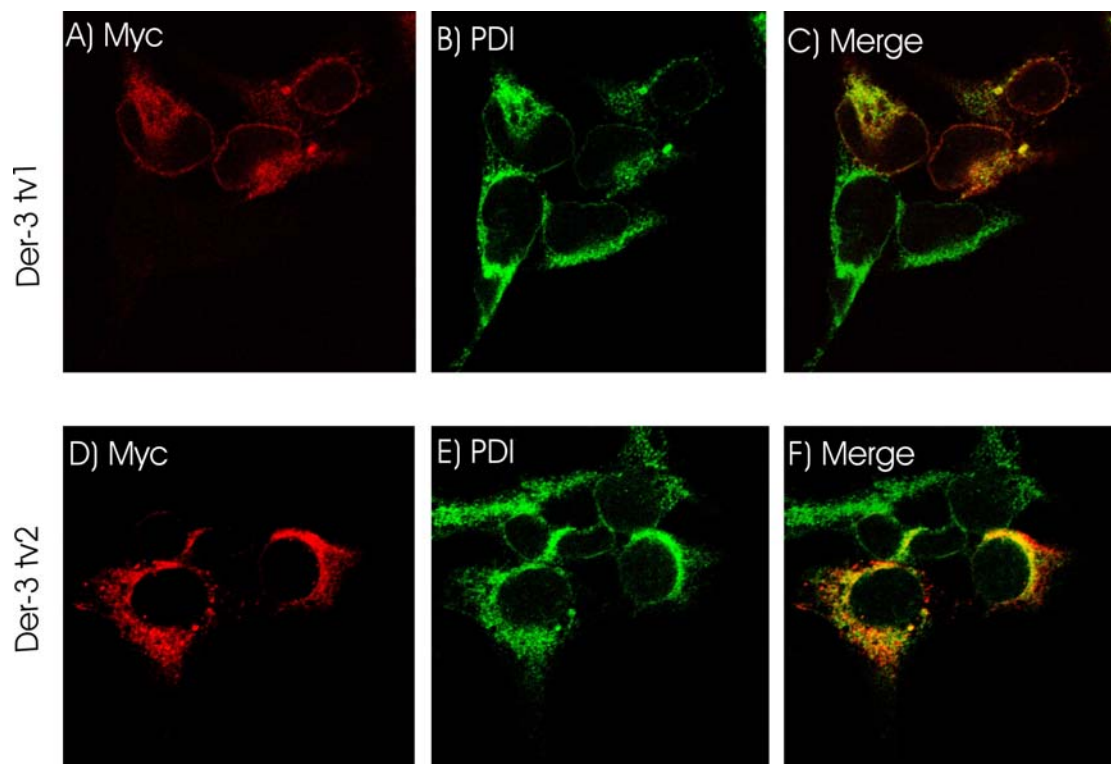


Figure 1: Confocal microscopy studies. Cells were transfected with a plasmid containing a myc-tagged version of both of Derlin-3 -tv1 and -tv2. The cells were then fixed and stained with a mouse anti-myc antibody and a rabbit anti-PDI antibody and visualised by a Rhodamine-labeled anti-mouse antibody (red channel) and CY2-labeled anti-rabbit antibody (green channel) respectively. When merged, colocalization is seen as a yellow colour.

Immunofluorescence analysis of transfected cells revealed that Derlin-3 tv1 and tv2 were colocalised with PDI (**figure 1**). This confirms that both transcription variants of Derlin-3 are indeed ER-resident proteins.

Retrotranslocation of ricin A-chain to the cytosol is decreased upon overexpression of Derlin-3

Overexpression of Derlin-3 accelerates the degradation of NHK [11]. To investigate whether overexpression of Derlin-3 affects retrotranslocation of ricin A-chain from the ER to the cytosol, we used plasmids expressing short and long (tv1, tv2) Derlin-3 cDNA (see Materials and methods). As a control, an empty pCDNA-4 vector was used. Cells were transiently transfected (see Materials and Methods) and experiments were performed 48 hours post-transfection.

To investigate whether overexpression of Derlin-3 would result in any difference in ricin cytotoxicity, cells overexpressing both transcription variants of Derlin-3 were incubated with ricin for 3 hours before protein synthesis was measured. As shown in **figure 2**, overexpression of Derlin-3 did not give any significant difference in sensitivity towards ricin. However, one should note that small differences are difficult to detect using this method.

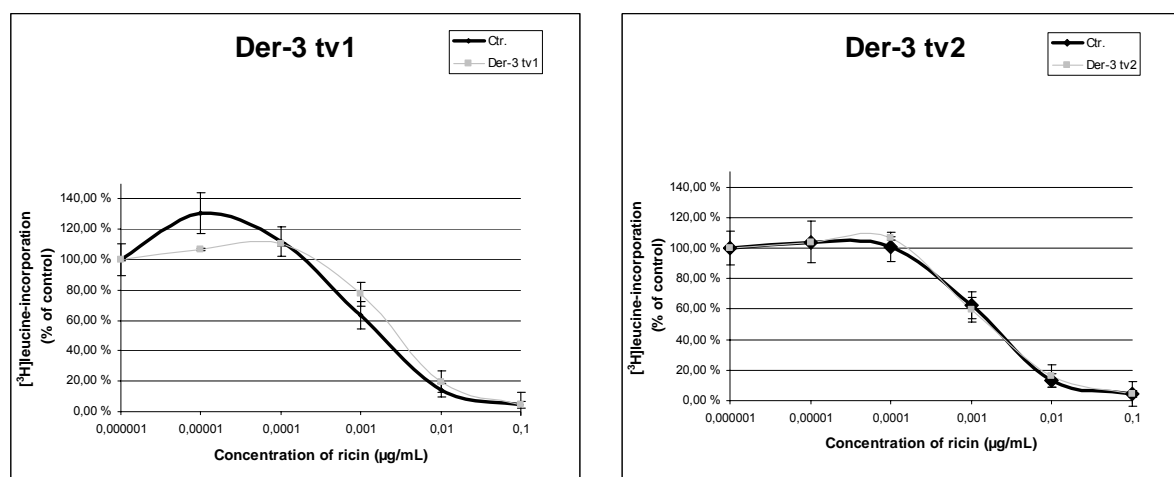


Figure 2: Effect of high expression of Derlin-3 on ricin cytotoxicity. Cells transiently transfected with Derlin-3 tv1 and tv2 and an empty control-vector, were incubated with increasing amounts of ricin in leucine-free medium. The medium was replaced with a Hepes medium containing [³H]leucine. Proteins were precipitated and proteins synthesis was measured as incorporated [³H]leucine using a β -counter. Protein synthesis was plotted in response to amount of ricin added. One representative experiment is shown.

To further investigate a possible role of Derlin-3 in retrotranslocation of ricin to the cytosol, we performed ricin sulf-1 sulfation-permeabilisation experiments (see Materials and Methods) in cells overexpressing Derlin-3 tv1 and tv2. We observed a

20% reduction in sulfated ricin sulf-1 A-chain in the cytosolic fraction of cells overexpressing both transcription variants of Derlin-3 (**figure 3**). These results indicate that the retrotranslocation of ricin A-chain to the cytosol is slightly reduced when Derlin-3 is overexpressed.

We saw a 10% reduction in general protein sulfation (data not shown) upon overexpression of Derlin-3 compared to control cells, which was not caused by a reduced amount of cellular proteins (data not shown). However, Derlin-3-transfected cells and control cells contained equal amounts of sulfated ricin in membrane fractions. It should be noted that overexpression of Derlin-3 gave a 5-10% reduction in sulfated holotoxin in the cytosolic fraction (data not shown).

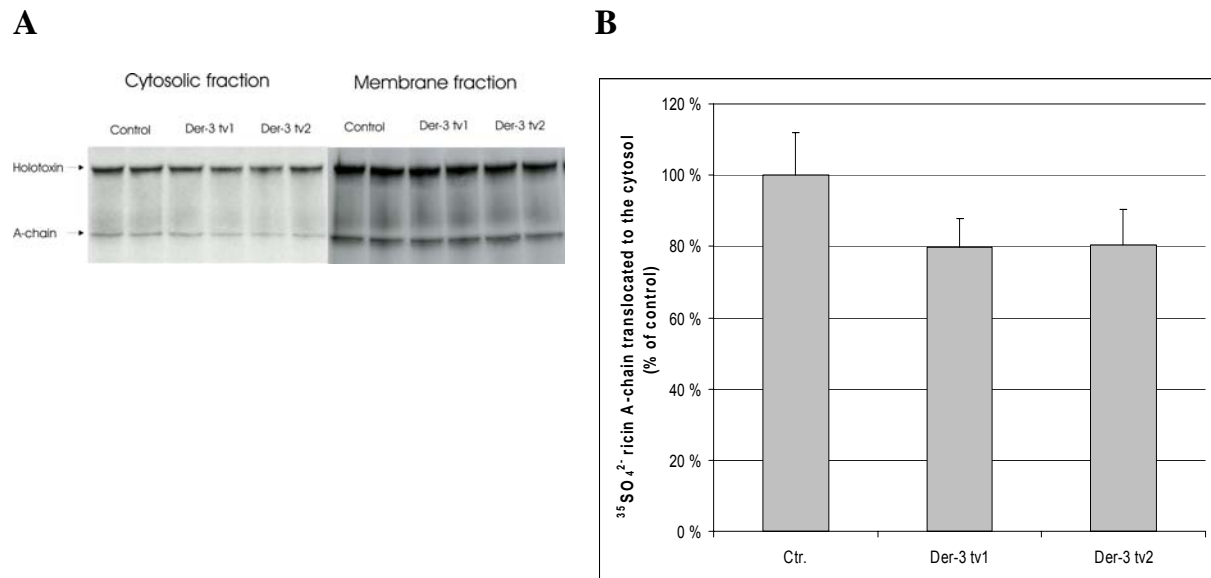


Figure 3: Effect of high expression of Derlin-3 on transport of ricin A-chain to the cytosol. Cells transiently transfected with constructs expressing Derlin-3 tv1 and tv2 and an empty control-vector were incubated with radioactive sulfate and ricin sulf-1. Cell-surface bound ricin sulf-1 was removed using 0.1M lactose. The cells were then permeabilised with digitonin and the cytosolic fraction was collected. The remaining fraction was lysed and collected. Ricin sulf-1 was immunoprecipitated and subjected to 12% SDS-PAGE under non-reducing conditions and the bands were visualised by autoradiography **A**) One representative gel is shown. **B**) The intensities of the bands were quantified using ImageQuant Tool version 5.0. The diagram represents data from three individual experiments. Error-bars indicate standard-deviations between the experiments.

Downregulation of endogenous Derlin-3 using RNA-interference

To investigate the involvement of Derlin-3 in the retrotranslocation of ricin to the cytosol we wanted to down-regulate the amount of endogenous protein by using RNAi. Two methods of RNAi were applied, namely the oligo-based approach and the vector-based approach. The design of oligos and vector-based constructs is described in Materials and Methods.

To verify knock-down of Derlin-3 expression, Western immunoblot analysis was performed on cell-lysates. The siRNAs were co-transfected with a plasmid expressing Derlin-3-tv2 cDNA fused with a myc-tag (see Materials and methods). An empty pCDNA4 vector was used as a control for the vector-based siRNAs. Mock siRNA was used as a control for the oligo-based siRNAs.

The vector-based siRNAs gave a much more efficient knock-down than the oligo-based siRNAs (**figure 4**), and we therefore decided to use the vector-based siRNAs in further experiments.

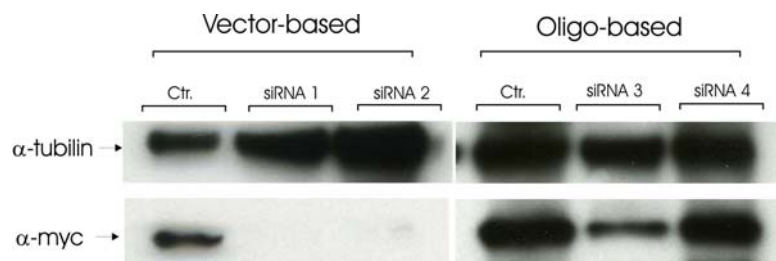


Figure 4: A Western blot analysis to determine the knock-down effect of the vector- based and the oligo-based siRNA respectively. Cells were co-transfected with the siRNAs together with a plasmid expressing a myc-tagged version of long Derlin-3. Cell-lysates were harvested after 48 hours and investigated by 12% SDS-PAGE followed by Western immunoblot analysis probing with an anti-myc antibody and anti- γ -tubulin for loading control.

Reduced expression of Derlin-3 decreases retrotranslocation of ricin A-chain to the cytosol

RNAi against Derlin-3 has been shown to block the degradation of the NHK mutant [11]. We therefore wanted to investigate whether reducing the amount of Derlin-3 would affect the sensitivity towards ricin. This was performed by using vector-based siRNA against Derlin-3 followed by measurement of protein synthesis.

Cells were transiently transfected with the constructs expressing siRNA against Derlin-3 (see Materials and Methods) and an empty pGESH vector as control. 48 hours post-transfection, cells were incubated with different concentrations of ricin before measurement of protein synthesis. **Figure 5** shows that reducing the expression of Derlin-3 does not affect ricin cytotoxicity. However, as mentioned earlier, small differences can be difficult to detect by this method.

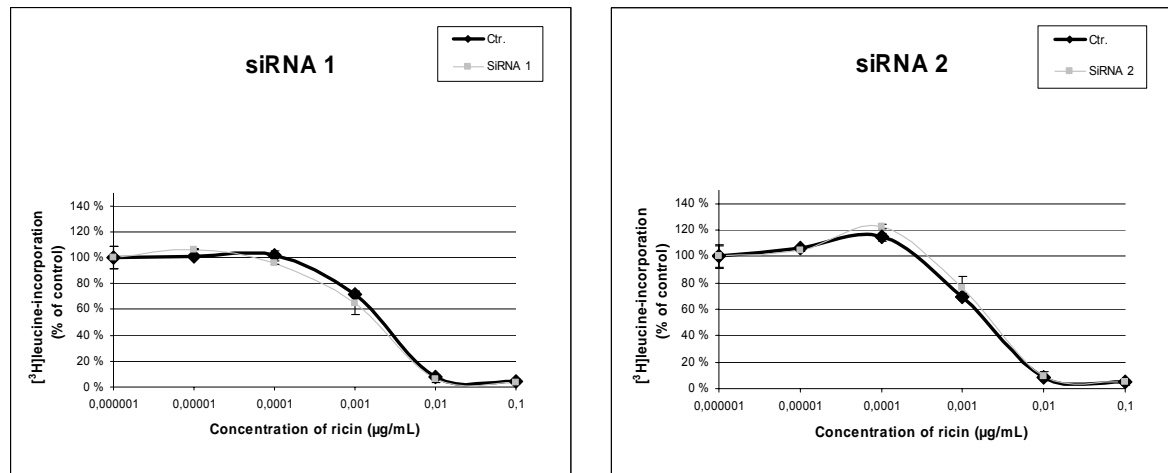


Figure 5: Effect of reduced expression of Derlin-3 on ricin cytotoxicity. Cells transiently transfected with constructs expressing siRNA against Derlin-3 and an empty control-vector, were incubated with increasing amounts of ricin in leucine-free medium. The medium was replaced with a HEPES medium containing [3 H]leucine. Proteins were precipitated and protein synthesis was measured as incorporated [3 H]leucine using a β -counter. Protein synthesis was plotted in response to amount of ricin added. One representative experiment is shown.

We then performed ricin sulf-1 sulfation and permeabilisation experiments (see Materials and Methods) on cells with reduced expression of Derlin-3. To knock down Derlin-3 expression, cells were transfected with the same constructs as in the cytotoxicity experiments. Experiments were performed two days post-transfection.

We observed a 20% reduction in sulfated ricin sulf-1 A-chain in the cytosolic fraction of cells where the expression of Derlin-3 was reduced using siRNA 1. In cells transfected with siRNA 2, we saw a 30% reduction in sulfated A-chain. These results indicate that reducing the expression of Derlin-3 gives a decrease in transport of ricin A-chain to the cytosol. This is consistent with the results that show a block in the degradation of NHK upon knock-down of Derlin-3 [11]. We also observed 15% reduction in amount of sulfated holotoxin in the cytosolic fraction compared to

control cells (data not shown). Control experiments showed that sulfation of cellular proteins was normal.

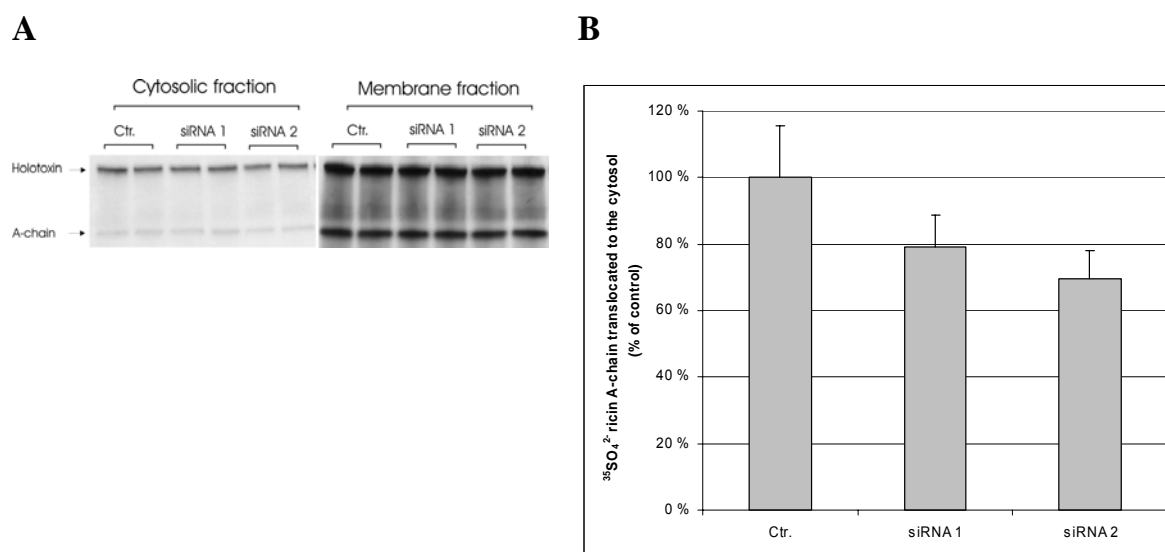


Figure 6: Effect of reduced expression of Derlin-3 on transport of ricin A-chain to the cytosol. Cells transiently transfected with vector-based siRNA against Derlin-3 and an empty control-vector were incubated with radioactive sulfate and ricin sulf-1. Cell-surface bound ricin sulf-1 was removed using 0.1M lactose. Cells were then permeabilised with digitonin and the cytosolic fraction was collected. The remaining fraction was lysed and collected. Ricin sulf-1 was immunoprecipitated and subjected to 12% SDS-PAGE under non-reducing conditions and the bands were visualised by autoradiography **A)** One representative gel is shown. **B)** The intensities of the bands were quantified using ImageQuant Tool version 5.0. The diagram represents data from three individual experiments. Error-bars indicate standard-deviations between the experiments.

Discussion

The present study indicates that Derlin-3 might be involved in retrotranslocation of ricin to the cytosol. In our study, HEK293 cells were made to overexpress Derlin-3 by transfecting the cells with myc-tagged Derlin-3. We confirmed that Derlin-3-myc, as shown previously [11], is localised in the ER. The protein was found to colocalise with the ER chaperone PDI. Our results demonstrate that overexpression of Derlin-3 decreases retrotranslocation of ricin A-chain to the cytosol by about 20%. In contrast to our results, overexpression of Derlin-3 has previously been reported to accelerate the degradation of the NHK mutant of the secretory glycoprotein $\alpha 1$ -antitrypsin [11]

indicating that more of the misfolded substrate is transported to the cytosol. There is a possibility that the observed decrease in retrotranslocation of ricin A-chain is due to an increased flow of misfolded proteins to the Sec61 translocon. Increased expression of Derlin-3 might increase the flow of proteins to undergo ERAD, thereby inhibiting the access of ricin to the translocon. This scenario has previously been reported for the chaperone EDEM [21]. A possible way to examine whether this is also the case when Derlin-3 is overexpressed might be to treat the cells with puromycin which is an inhibitor of protein synthesis, thus keeping the translocation channels open [22].

It should be noted that the sulfated ricin A-chain present in the cytosolic fraction analysed in the sulfation-permeabilisation experiments does not represent the total amount of A-chain translocated to the cytosol. Low concentrations of digitonin had to be used to avoid leakage from other organelles. However, this released only 20–25% of ricin to external the cytosolic fraction. Thus, sulfated ricin A-chain present in the “membrane fraction” not only consists of ricin A-chain in the ER, but also of ricin A-chain partially or completely translocated to the cytosol.

The Derlin-proteins may require additional factors to transport substrates across the ER-membrane. Experiments have shown that Derlin-1 and -2 form a multiprotein complex with other ERAD components [23]. Furthermore, Derlin-2 and -3 are associated with EDEM [11]. When studying the effects of overexpression, one has to take into account the limited amount of interaction partners.

Reduced expression of Derlin-3 gave an approximate 25% decrease in the amount of sulfated ricin A-chain in the cytosol. Knock-down of Derlin-3 by RNAi has previously been demonstrated to block the degradation of misfolded glycoproteins [11]. However, the low lysine-content in ricin reduces the risk of degradation by the proteasomes [24]. It is important to note that results obtained with RNAi should be approached with caution. Proteins have different half-lives, and due to the lack of an antibody against endogenous Derlin-3, knock-down efficiency could not be tested for each individual experiment. Investigation of mRNA levels could be achieved by real-time reverse transcriptase-polymerase chain reaction (Real-time PCR). However, an

efficient reduction in mRNA-levels would not exclude that residual protein might be present due to a long half-life of the protein in the cell. Furthermore, the downregulation of one pathway might lead to the upregulation of another. Derlin-2 has 70% sequence similarity to Derlin-3, and these two proteins seem to constitute a functional group different from Derlin-1 [11]. It is plausible that Derlin-2 might be upregulated in response to a downregulation of Derlin-3. An example of compensatory mechanisms is shown in HeLa cells where the loss of clathrin-dependent endocytosis is compensated by inducing an alternate endocytic pathway [25].

The homeostasis of the endoplasmic reticulum (ER) is tightly regulated and accumulation of misfolded proteins in the ER lumen induces a cellular response known as the ER stress response or unfolded protein response (UPR). The UPR increases the expression of a large number of chaperones involved in protein folding and also decreases the flow of proteins into the ER by halting translation [26-28]. Use of RNAi against Derlin-1 in *Caenorhabditis elegans* has been shown to evoke ER-stress [29]. Thus, the manipulation of Derlin-3 expression levels might lead to a range of secondary responses. However, a reduced transport of newly synthesised proteins into the ER due to a halt in translation should rather decrease the competition between ricin and ERAD substrates in the transport out of the ER. Thus, we would expect to see an increase in ricin A-chain in the cytosol, rather than a decrease.

It should be noted that reduced expression and overexpression of Derlin-3 gave a reduced presence of holotoxin in the cytosol. The general belief is that only the A-chain of ricin is retrotranslocated to the cytosol. However, we observe a band representing sulfated holotoxin in the cytosolic fraction in our experiments. It seems that Derlin-3 might be involved in retrotranslocation of both holotoxin and A-chain from the ER to the cytosol. However, the effect of Derlin-3 on retrotranslocation of holotoxin seems to be smaller than that of the A-chain.

Ricin has been shown to interact with the Sec61 complex [30]. It has been assumed that it is the main pathway by which ricin reaches the cytosol. However, one might

hypothesise that ricin is able to use other channels or translocators for its transport to the cytosol. It is not known whether the Derlin proteins work as accessory proteins for the Sec61 or if they are able to form a protein-conducting channel of their own. Derlin proteins might form multiprotein complexes together with several other components implicated in ERAD [5,29]. In future studies of ricin trafficking, it would be interesting to clarify whether ricin indeed interacts with Derlin-3 in the ER. It would also be of interest to knock down the expression of other components that interact with Derlin-3, such as for instance EDEM. Even though Derlin-3 seems to be involved in retrotranslocation of ricin, we can not draw any final conclusion until further studies have been performed.

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APPENDIX I

RETROTRANSLOCATION OF RICIN HOLOTOXIN FROM THE ENDOPLASMIC RETICULUM TO THE CYTOSOL

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Abstract

The plant toxin ricin is a member of the family of AB-toxins, meaning that it consists of two moieties, an A-moiety that has enzymatic activity and a B-moiety which binds to the cell surface. After binding and endocytosis, toxin is taken up by the cell and follows a retrograde pathway through the *trans*-Golgi Network (TGN) to the endoplasmic reticulum (ER) before it is retrotranslocated to the cytosol. Ricin consists of a 32 kDa A-chain and a 34 kDa B-chain that is held together by a disulfide-bond. The disulfide-bond connecting the two chains can be reduced in the ER, and the A-chain is, after retrotranslocation to the cytosol, able to inactivate ribosomes. It is, however, not known if reduction of the toxin is required prior to translocation or whether intact toxin (holotoxin) is also translocated followed by reduction in the cytosol.

The present study suggests that ricin holotoxin is retrotranslocated to the cytosol, and that this might occur by the same mechanisms as translocation of ricin A-chain.

Introduction

The protein toxin ricin is produced in the seeds of the castor oil plant *Ricinus Communis*. The toxin is able to disrupt protein synthesis by binding to and inactivating ribosomes [1,2]. Ricin consists of an A- and a B-chain of approximately 30 KDa each that are connected by a disulfide-bond. The B-chain is a lectin and is able to bind to galactose containing surface receptors. After binding, the whole toxin is endocytosed and follows a retrograde pathway to the endoplasmic reticulum (ER) via the *trans*-Golgi network (TGN).

Proteins are imported in a co-translational manner into the ER via the Sec61/ribosome complex. In the ER lumen, nascent peptides are subjected to a range of components that ensure proper folding. However, some proteins do not obtain the correct folding and have to be retrotranslocated to the cytosol for degradation by the proteasomes in a process known as ER-associated degradation (ERAD) [3,4]. The Sec61 translocon was suggested to be involved in retrotranslocation when it was observed that ERAD substrates were coimmunoprecipitated with this protein-conducting channel [5]. Ricin is believed to exploit the ERAD pathway to gain access to the cytosol [6,7] and to translocate through the Sec61 [8,9].

The disulfide-bond connecting the A- and the B-chain can be reduced in the ER by protein disulfide isomerase (PDI) [10] and thioredoxin reductase [11] before the A-chain is partially unfolded and retrotranslocated to the cytosol. For ricin to exert its toxic effect, the A-chain must be reductively cleaved from the B-chain to release a steric block of the active site [12,13]. However, it is not known whether this reducing event is absolutely required for toxin retrotranslocation, or if intact toxin (holotoxin) can be retrotranslocated to the cytosol. Recent results from our lab have shown that holotoxin can be detected in the cytosol. We have also shown that holotoxin is able to bind to the Sec61 translocon [9]. Furthermore, a toxin with a non-reducible bond has been shown to be equally cytotoxic as native ricin [14] indicating that holotoxin might somehow be transported to the cytosol.

The present study is aimed at investigating retrotranslocation of ricin holotoxin from the ER to the cytosol. By permeabilising cells with digitonin, we are able to separate the cytosolic fraction from the membrane-containing fraction, and thereby investigate the amount of ricin A-chain and holotoxin present in the cytosol.

Materials and methods

Materials

Reagents and inhibitors: Hepes, lactose, Dithiothreitol (DTT), digitonin and N-Ethylmaleimide (NEM) were all obtained from Sigma Chemical Co. (St Louis, MO, USA). $\text{Na}_2^{35}\text{SO}_4$ is manufactured by Montebello diagnostics (Montebello diagnostics, 0379 Oslo, Norway).

Antibodies: Rabbit polyclonal anti-ricin antibodies were from Sigma Chemical Co (St Louis, MO, USA). Mouse anti-calnexin (BD Biosciences, Palo Alto, CA, USA), anti-calreticulin (BioSite, San Diego, CA, USA) and anti- γ -tubulin antibodies (Sigma Chemical Co., St Louis, MO, USA) and rabbit anti-PDI antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) were used for Western immunoblotting. The secondary antibodies horseradish peroxidase (HRP)-linked goat anti-mouse and HRP-linked goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) were used in Western immunoblotting analysis.

Cell culture

Human Embryonic Kidney-cells (HEK 293) are an epithelial cell-line derived originally from embryonic kidney. These cells were used in all experiments. Cells were grown in Falcon cell culture flasks (BD Biosciences, Palo Alto, CA, USA) and maintained in Dulbecco's Modified Eagle Medium DMEM (BioWhittaker, MD, USA) supplemented with 10% fetal calf serum (FCS) (Integro b.v, Zaandam, Netherlands), 100 units/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (Invitrogen,

Carlsbad, CA) under 5% CO₂ in a 37°C incubator. The cells were seeded on Falcon cell culture plates coated with poly-L-lysine (Sigma Chemical, St Louis, MO).

Sulfation of ricin sulf-1 and permeabilisation of cells

Ricin A-chain sulf-1, containing a tyrosine sulfation site was produced, purified and reconstituted with ricin B-chain to form ricin sulf-1 [15]. After approximately 3 hours, the toxin reaches the ER where it is retrotranslocated to the cytosol. To measure transport to the cytosol, the cells are treated with low concentrations of digitonin to permeabilise the plasma membrane, thereby releasing the cytosolic fraction.

Cells were incubated for 3 hours in sulfate-free medium containing 1% non-essential amino-acids (Bio Whittaker) and 0,2mCi/mL Na₂³⁵SO₄ before ~500ng/mL ricin sulf-1 was added, and the incubation was continued for 3 hours at 37°C. The medium was removed and cells were washed twice at 37°C with a solution of 0.1M lactose in HEPES-medium, to remove surface-bound ricin. Cells were then washed twice with room-tempered PBS and permeabilised in a KOAc-buffer (115 mM CH₃COOK, 25 mM HEPES, and 2.5mM MgCl₂, pH 7.4) containing 3µg/mL digitonin. The cells were first incubated at room-temperature for 5 minutes, followed by 30 minute incubation on ice allowing cytosolic proteins to leak out of the cells. The supernatant was removed and a lysis-buffer (lysis-buffer: 0.1M NaCl, 10mM Na₂HPO₄, 1mM EDTA and 1% Triton X-100, pH 7.4) in the presence of a protease inhibitor (Roche diagnostics, Mannheim, Germany) was added to the remaining fraction (membrane-containing fraction). Both fractions were spun down for 5 minutes at 5000 rpm in an eppendorf centrifuge (Eppendorf, Hamburg, Germany) to remove debris and nuclei.

Immunoprecipitation of ricinsulf-1 was done using protein-A-sepharose CL-4B (GE Healthcare, Princeton, NJ, USA) coated with rabbit-anti-ricin antibodies overnight at 4°C. After binding of sulfated ricin, the beads were washed with a 0.35% Triton X-

100: PBS solution. A non-reducing loading buffer was added to the beads before boiling at 100°C to release bound material. SDS-PAGE was performed using a 12% gel, and the proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Dried membranes were exposed to Kodak BioMax MR film (Rochester, NY, USA) at room temperature. Signal intensities of the bands were quantified using ImageQuant Tool version 5.0 (GE Healthcare, Princeton, NJ, USA). The slowest migrating band of 60 kDa represents intact toxin (holotoxin), and the fastest migrating band of 30 kDa is ricin A-chain.

Cell lysates were precipitated in 5% TCA and dissolved in 0.1M of KOH before the radioactivity was measured on a β -counter. The radioactivity measured in the precipitates represents total protein sulfation. This is done as a control to detect possible differences in the total amount of isotope incorporated under the different conditions.

Western immunoblot analysis

Cells were washed twice with cold PBS before lysis in lysis-buffer (0.1M NaCl, 10mM Na₂HPO₄, 1mM EDTA and 1% Triton X-100, pH 7.4) supplemented with complete protease inhibitors (Roche Diagnostics, Basel, Switzerland). Lysate was harvested by using a cell-scraper and transferred to eppendorf-tubes before centrifugation for 5 minutes at 4000 rpm at 4°C to remove cell debris. The supernatant was added a reducing buffer and boiled for 5 minutes at 100°C. Proteins were then analyzed by 12% SDS-PAGE and subsequently electroblotted onto a PVDF-membrane. The membrane was blocked using PBS/0.05% Tween-20 with 5% of non-fat dried milk. All antibodies were diluted in 5% non-fat dried milk in PBS with 0.1% Tween-20. SuperSignal West Pico Chemiluminescent substrate or SuperSignal West Femto Chemiluminescent substrate (Pierce, Rockford, IL) was used for immunodetection.

Results

Permeabilisation of plasma membrane with digitonin does not cause leakage from the ER

To measure toxin transport to the cytosol, the cells were permeabilised using the mild detergent digitonin, which binds specifically to cholesterol and induces the formation of permanent holes in the membrane [16]. The plasma membrane has a much larger content of cholesterol than intracellular membranes, and if an appropriate concentration of digitonin is used, this leaves organelles more or less intact. To determine the optimal concentration of digitonin that does not give a leakage from organelles such as the ER, a sulfation-permeabilisation experiment was performed with digitonin-concentrations ranging from 0 to 8µg/mL.

As seen in **Figure 1 A**, there was an increase in both ricin A-chain and ricin holotoxin in the fraction released from cells upon increasing concentrations of digitonin. Interestingly, even in the cytosolic fraction without digitonin, there was a considerable amount of ricin present. This might be due to rupture of the plasma membrane of cells during the experiments. Permeabilisation of cells with increasing concentrations of digitonin gave a gradual increase in both holotoxin and A-chain (**figure 1 B**). Ricin can be reduced in the ER [10,11], and this might indicate that no holotoxin has leaked from the Golgi upon increased concentrations of digitonin.

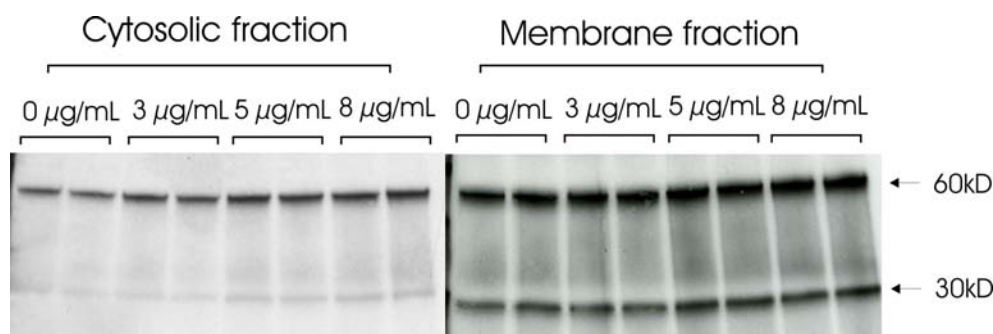
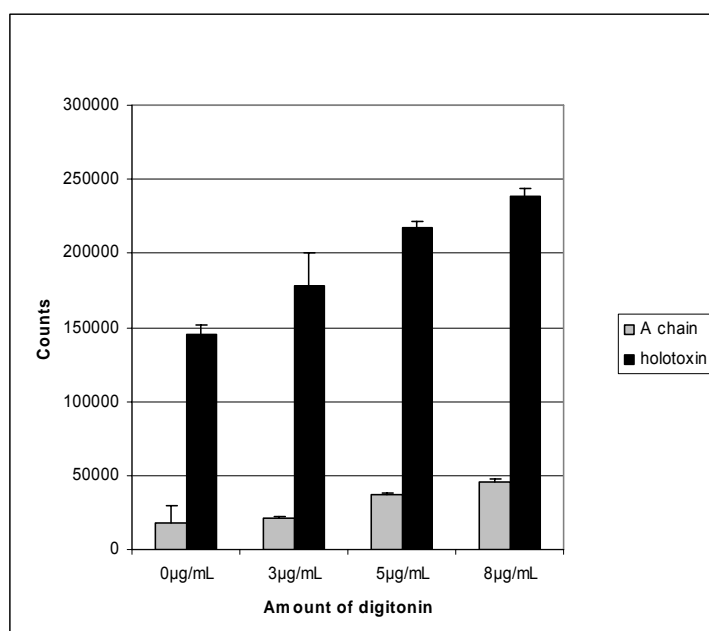
A**B**

Figure 1: Permeabilisation of cells with increasing amount of digitonin. Cells were incubated with radioactive sulfate and ricin sulf-1. Cell-surface bound ricin sulf-1 was removed using 0.1M lactose. The cells were then permeabilised with digitonin and the cytosolic fraction was collected. The remaining fraction was lysed and collected. Ricin sulf-1 was immunoprecipitated and subjected to 12% SDS-PAGE under non-reducing conditions and developed by autoradiography **A)** One representative gel is shown. **B)** The intensities of the bands were quantified using ImageQuant Tool version 5.0. The diagram represents data from three individual experiments. Error-bars indicate standard-deviations between the experiments.

The ER has a relatively low concentration of cholesterol, however one can not exclude that the digitonin-treatment might have caused some leakage from this organelle. Cell-lysates were therefore harvested and investigated by Western immunoblotting to determine whether there was any leakage of the ER-resident proteins calnexin (CNX) and calreticulin (CRT). **Figure 2** shows

that neither CNX nor CRT was present in the cytosolic fraction, indicating that the ER was kept intact after the digitonin-treatment.

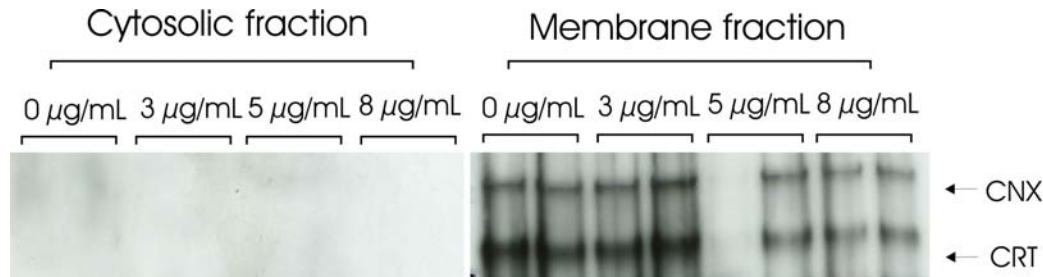


Figure 2: Western immunoblot analysis to determine possible leakage from the ER. Cell lysates from the sulfation-permeabilisation experiment were precipitated with 5% TCA on ice for 30 minutes. The supernatant was removed and the pellet was incubated with ether overnight. The ether was left to evaporate, and the pellet was dissolved in a reducing sample-buffer at 100°C for 20 minutes. Proteins were investigated by 12% SDS-PAGE under reducing conditions followed by Western immunoblot analysis probing with mouse anti-calnexin antibodies and rabbit anti-PDI antibodies.

DTT increases translocation of ricin A-chain to the cytosol

To investigate whether an increased reduction of ricin would facilitate the translocation of the A-chain to the cytosol, a sulfation was performed. During the last 30 minutes of incubation with ricin sulf-1, different concentrations of the reducing agent DTT (0-100mM) were added to the cells. Cells were permeabilised with digitonin to allow cytosolic proteins to leak out. **Figure 4** shows that the release of A-chain to the cytosol correlated well with the increasing amounts of DTT added. It is interesting to note that even in the fractions with 100mM of DTT the holotoxin was still present in the cytosol, indicating that even high amounts of DTT are still not able to reduce ricin completely.

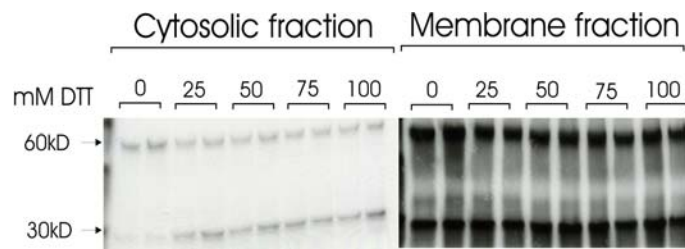
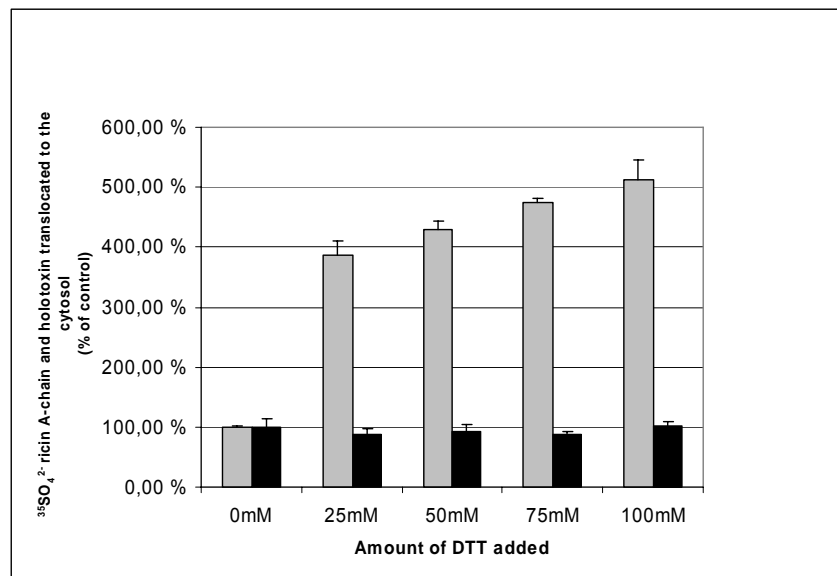
A**B**

Figure 3: Translocation of ricin A-chain and holotoxin to the cytosol upon increasing concentration of DTT. Cells were incubated with radioactive sulfate and ricin sulf-1 and then treated with increasing amounts of digitonin. Cell-surface bound ricin sulf-1 was removed using 0.1M lactose before incubation in a KOAc-buffer with 3µg/mL digitonin and 1% NEM. The supernatant was removed (cytosol fraction) and the remaining fraction was lysed in a lysis-buffer supplemented with 1% NEM (membrane fraction). Ricin sulf-1 was immunoprecipitated and subjected to 12% SDS-PAGE under non-reducing conditions. The bands were visualised by autoradiography **A**) One representative gel is shown. **B**) The intensities of the bands were quantified using ImageQuant Tool version 5.0. The diagram represents data from three individual experiments. Error-bars indicate standard-deviations between the experiments.

Discussion

The present study suggests that ricin holotoxin is retrotranslocated to the cytosol. Digitonin induces the formation of holes in the cholesterol-rich plasma membrane and is thought to leave organelles more or less intact. It is highly relevant to ask whether the sulfated holotoxin we observe in the cytosol is due to a leakage from the Golgi apparatus. However, we do not think that this is the case since the amount of both holotoxin and A-chain increased with the amount of digitonin. A more precise way to show this would be to perform Western immunoblot analysis probing for a soluble Golgi resident protein in the cytosolic fraction from sulfation-permeabilisation experiments. However, due to the lack of such a protein, we have so far not been able to perform such an analysis. An alternative approach is to transfect cells with a tagged version of a protein we know is transported to the Golgi apparatus, such as the proteoglycan serglycin [17] and then test for leakage. The source of the holotoxin does not seem to be vesicles transported from the TGN to the plasma membrane (data not shown).

We have also shown that increased reduction of ricin with DTT clearly augmented the retrotranslocation of ricin A-chain to the cytosol. However, even high concentrations of DTT (100mM) were not able to reduce ricin completely.

A possible way to determine whether the holotoxin is indeed retrotranslocated from the ER could be to perform sulfation-permeabilisation experiments on ricin sulf-2. This molecule contains three partly overlapping N-glycosylation sites in the A-chain [15]. Since core glycosylation of proteins occurs in the ER lumen, this provides a way to study transport to this organelle. However, results obtained with this method were not quantifiable due to extremely low signals in the cytosolic fraction (data not shown).

Ricin is believed to utilise the Sec61 translocon for transport to the cytosol [8]. It has been suggested that in order to be transported through this pore, the holotoxin must be reduced and the A-chain partially unfolded. The actual size of the pore has not

been clarified completely and sizes from ~ 5 Ångström (or 0.5 nm) to as much as 60 Ångström have been suggested [18]. It is debatable whether the holotoxin would be small enough to fit into this pore. However, there is a possibility that ricin may use other mechanisms to gain access to the cytosol which does not involve translocation through Sec61. Recently, a vesicular budding pathway out of the ER was discovered that did not involve COPII exit sites. [19]. EDEM was found to be sequestered into these vesicles together with NHK and Derlin-2, indicating an additional pathway for clearance of misfolded from the ER.

Clearly, more research is required to clarify the retrotranslocation process of ricin. The possibility still exists that other mechanisms for retrotranslocation could be utilised, since several components necessary for transport of certain toxins is not required for ricin transport. It is therefore plausible that ricin could be able to reach the cytosol by different and yet unresolved mechanisms.

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